Mechanism and efficacy of a GD2-specific immunotherapy using NK cells

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Abbreviations

13-cis-RA	13-cis-retinoic acid
4-HPR	N-(4-hydroxyphenyl)retinamide
ADCC	antibody-dependent cellular cytotoxicity
anti-IdAb	anti-idiotype antibody
BAT3	HLA-B associated transcript 3
BSA	bovine serum albumin
CAR	chimeric antigen receptor
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity
CRS	cytokine release syndrome
DAPI	diaminophenylindole
DC	dendritic cell
DD	death domain
DFF	DNA-fragmentation factor
DISC	death-inducing signaling complex
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
DR	death receptor
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FADD	Fas-associated death domain adaptor protein
FBS	fetal bovine serum
GCS	glucosylceramide synthase
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practice
GvHD	graft-versus-host disease
HACA	human anti-chimeric antibody
HAMA	human anti-mouse antibody
HLA	human leukocyte antigen

HRP	horseradish peroxidase
HSCT	hematopoietic stem cell transplantation
i.p.	intraperitoneal
IC	immunocytokine
iCAR	inhibitory chimeric antigen receptor
IDRF	image-defined risk factors
IFN	interferon
Ig	immunoglobulin
IL	interleukin
INRG	International Neuroblastoma Research Group
INRGSS	International Neuroblastoma Research Group Stratification System
INSS	International Neuroblastoma Staging System
ITIM	immunoreceptor tyrosine-based inhibitory motif
KIR	killer cell immunoglobulin-like receptor
LAK	lymphokine activated killer
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MICA/B	MHC class I-related chain
MRD	minimal residual disease
mRNA	messenger ribonucleic acid
NB	neuroblastoma
NCR	natural cytotoxicity receptor
NK	natural killer
NSG	NOD scid gamma
PBS	phosphate buffered saline
PE	phycoerythrin
PI	propidium iodide
PMA	phorbol myristate acetate
PPPP	1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol
PVR	poliovirus receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature

s.c.	subcutaneous
scFv	single chain fragment variable
SDS	sodium dodecyl sulfate
TAA	tumor-associated antigen
TCR	T cell receptor
TEMED	tetramethylethylendiamine
TGF	transforming growth factor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
ULBP	UL16-binding protein

1. Introduction

1.1. Neuroblastoma

Neuroblastoma (NB) is an aggressive childhood malignancy that accounts for 8-10% of all childhood cancers [1] and approximately 15% of all pediatric oncology deaths [2]. It is a solid, extracranial tumor that originates from neural crest cells of the sympathetic nervous system and consists of undifferentiated, neuroectodermal cells. In general, NB tumors can be located at any side along the sympathetic chain, such as neck, chest or pelvis, but the most common location of primary tumor growth is abdominal, generally within the adrenal glands [3]. The clinical appearance of NB is heterogeneous, as patients may experience spontaneous regression or differentiation while others endure aggressive growth and rapid spread of the disease. To account for this wide variability in clinical behavior, staging systems have been established to assign patients to different risk groups with the purpose of avoiding overtreatment among low-risk patients while intensifying therapy for patients identified as high-risk. The International Neuroblastoma Staging System (INSS) assigns patients to different groups, based on their age at time of diagnosis, extent of the disease and the resectability of the tumor [4, 5]. Since this staging system is based on a post-surgical evaluation, the International Neuroblastoma Research Group (INRG) established a new pre-treatment risk stratification system (INRGSS) to improve reproducibility of clinical trial results. This INRG staging system is based on image-defined risk factors (IDRF), age and biological prognostic factors, such as MYCN-amplification, chromosomal 11q aberrations or DNA ploidy [6]. About 20% of NB patients exhibit an amplification of the oncogene MYCN, which is associated with rapid progression of the disease and poor prognosis [7, 8]. Deletion of chromosome 11q is associated with decreased event-free survival [9]. DNA ploidy can be used as a prognostic factor for infants with NB, for which diploidy of tumor cells was shown to be associated with advanced tumor stages and reduced responsiveness to chemotherapy [10, 11]. In summary, about 50% of all NB cases belong to the high-risk group [2], characterized by poor prognosis and a 5 year event-free survival rate of less than 50% despite intensive multimodal therapy [12].

1.2. Treatment of neuroblastoma

Since clinical behavior of NB is very heterogeneous, treatment of NB varies widely depending on the risk group to which patients are initially assigned. The treatment of low-risk NB includes observation only (generally in the special case of INSS stage 4S) or moderate chemotherapy. In case of intermediate-risk patients, treatment is based on

surgical removal of the tumor, followed by moderate multi-agent chemotherapy [5]. The treatment of high-risk patients is the most challenging and can be divided into induction therapy, consolidation therapy and maintenance therapy. Induction therapy aims at reducing the tumor burden and is based on high dose multi-agent chemotherapy as well as irradiation of the primary tumor site. Consolidation therapy includes removal of the primary tumor and metastases. Further high-dose myeloablative chemotherapy, followed by rescue with autologous hematopoietic progenitor cells is currently used as consolidation therapy in most of the high-risk clinical trials [5, 13-15]. Although the majority of high-risk patients initially respond to induction and consolidation therapy, they often develop progressive disease or relapse. These relapses are potentially caused by minimal residual disease (MRD) consisting of cells that have acquired drug resistance or that have been otherwise selected from a heterogeneous population of tumor cells during induction and consolidation therapy [12, 16]. Therefore, maintenance therapy aims at eradication of minimal residual disease that could potentially cause a relapse. The standard treatment of minimal residual disease is based on the application of 13-cis-retinoic acid (Isotretinoin) [17, 18]. Retinoic compounds are natural or synthetic derivates of vitamin A that are able to induce cell growth arrest or differentiation in NB cells [19]. Furthermore, the synthetic vitamin A derivate Fenretinide (N-(4-hydroxyphenyl)retinamide; 4-HPR) is under investigation for application in NB treatment [20, 21]. Fenretinide was demonstrated to be effective against NB cell lines, even if these cells were resistant to other retinoic compounds such as 13-cis-RA [22]. Mechanisms suggested to be involved in 4-HPR-mediated cytotoxicity include the intracellular accumulation of ceramides and production of reactive oxygen species (ROS) [23, 24].

Despite existing intensive multi-modal standard therapy, the outcome of high-risk NB patients remains poor, emphasizing the need for new and more effective approaches to treat minimal residual disease. Therefore, immunotherapeutic approaches are under current investigation and appear to be promising alternatives to address this critical problem.

1.3. Immunotherapy of neuroblastoma

Cancer immunotherapy aims at activating the immune system to specifically target and kill tumor cells. A variety of immunotherapeutic strategies is currently being investigated that can be divided into two broad categories. Passive immunotherapeutic approaches mediate an immediate but only transient effect. They are based on the direct application of immunostimulatory cytokines, such as interleukin-2 (IL-2) or monoclonal antibodies (mAbs), which specifically target a tumor-associated antigen (TAA). Additionally,

immune effector cells such as T cells or NK cells can be adoptively transferred for passive immunotherapy. In contrast, active immunotherapeutic strategies are utilized to induce a long-lasting anti-tumor effect by stimulating the immune system with the help of cancer vaccines. These cancer vaccines include peptide/protein vaccines, DNA vaccines or dendritic cell vaccines.

The susceptibility of a certain tumor entity to immunotherapy in general, as well as the selection of the most promising immunotherapeutic approach, depends on the immunogenicity of the tumor. An important prerequisite for immunogenicity is the expression and successful presentation of TAAs to induce a specific immune response. In case of NB, several TAAs have already been identified (Table 1.3) [25] confirming that NB can be recognized by the immune system.

Table 1.3.: Tumor-associated antigens expressed in human neuroblastoma tumors, adapted from Gray et al. [25].

Antigen	Nature of antigen	Tumor expression	Normal tissue expression
MYC-N	Transcription factor and proto-oncogen	Over-expression in >40% of patients with metastatic disease	Little expression beyond foetal development
Disialoganglioside (GD2)	Glycolipid	100% of NB	CNS neurons and peripheral pain fibers
Tyrosine hydroxylase	Catecholamine biosynthesis	100% of NB samples	Adrenal medulla and CNS dopaminergic neurons
Hu antigens	Neuronal-specific RNA binding proteins	80% of NB express HuD	Central and peripheral nervous tissue
Survivin	Inhibitor of apoptosis	Expression 26/26 high-risk NB	Low or absent in normal tissue
Melanoma antigen A (MAGE) family	Cancer germline antigens	8/10 NB express at least 1 MAGE family antigen	Little expression in normal tissue other than testis
NY-ESO-1	Cancer germline antigen	18/22 (82%) NB samples	Normal tissue expression restricted to testis
PReferentially expressed Antigen in MElanoma (PRAME)	Cancer germline antigen	87/94 (93%) NB samples	Expression in normal tissue restricted to testis
Anaplastic lymphoma kinase (ALK)	Receptor tyrosine kinase	Expression in 14/16 NB samples	Limited expression on neural tissue

Despite the expression of these TAAs, spontaneous immune responses in NB patients are generally weak and fail to effectively control tumor growth on their own [25]. Various factors have an impact on the induction of an effective anti-tumor response, such as the nature of the antigen or the development of immune escape mechanisms by the tumor. Furthermore, low expression of the antigen might not be sufficient to effectively induce a specific immune response. In the case of NB, the disialoganglioside GD2 is highly expressed on NB tumors. Gangliosides are sialic acid-containing glycosphingolipids. They consist of a hydrophobic ceramide, which is anchored in the cell membrane, and an extracellular hydrophilic oligosaccharide chain, which is connected to one ore more molecules of sialic acid [26]. GD2 is consistently expressed on neuroectodermal tumors, such as NB and most melanomas. Further, GD2 has been reported to be expressed on a variety of other tumor entities, such as bone and soft-tissue sarcomas, small cell lung cancer or brain tumors [27, 28]. Physiological expression of GD2 is restricted to the neurons, skin melanocytes and peripheral pain fibers [29]. So far, the exact function of GD2 is not completely understood, but it could be shown that GD2 together with GD3 is involved in the attachment of tumor cells to extracellular matrix proteins [30].

Due to its high expression in NB but restricted physiological expression, GD2 is a suitable TAA for immunotherapeutic approaches in NB treatment. Importantly, analysis of tumor samples derived from patients before and after treatment with anti-GD2 antibodies revealed that GD2 expression persisted in refractory or recurrent NB, indicating that GD2 is not modulated off the cell membrane during therapy [31]. This stable expression of GD2 on NB tumors is an important prerequisite for GD2-directed immunotherapies. As a glycolipid, GD2 is a T cell-independent antigen and cannot induce a GD2-specific T cell response. Therefore, GD2-directed therapeutic appoaches focus on passive immunotherapy with GD2-specific antibodies.

In addition to the nature of the TAA and level of its expression, the presentation of antigens in the context of MHC class I molecules is another factor that has an impact on the immunogenicity of a tumor. Antigen presentation can be negatively affected in several ways. This can be caused by defects in the antigen processing machinery as well as low or absent MHC class I expression. In case of NB, it is known that tumors exhibit low or absent MHC class I expression, thereby being unable to present TAAs to cytotoxic T cells [32]. The expression of antigen processing genes, another prerequisite for a successful presentation of the TAAs, is only low in NB [33, 34]. Although low or absent MHC class I expression negatively affects activation of cytotoxic T cells, this actually renders NB cells sensitive to recognition by NK cells. In general, NK cell-mediated lysis of tumor cells is

further promoted by expression of activating NK cell ligands on tumor cells, such as MICA/B, ULBP-1 or ligands for NCRs, which will be discussed in more detail in 1.4. In the case of NB, NCRs and the DNAX accessory molecule-1 (DNAM-1) have been shown to play an important role in NK cell-mediated lysis [35, 36]. Unfortunately, tumor cells have evolved mechanisms to escape recognition by NK cells. This includes either downregulation or release/shedding of activating NK cell ligands from the tumor cell surface. Further, the release of soluble activating ligands can result in downregulation of activating receptors, such as NKG2D, on effector cells [37-39]. Immunosuppressive cytokines, such as transforming growth factor-β (TGF-β), produced in the tumor microenvironment also negatively affect the expression of activating receptors as well as some of their ligands [40-42].

Based on the current knowledge about the immunogenicity of NB, various novel passive and active immunotherapeutic approaches specifically targeting NB are being evaluated. Active anti-NB strategies include dendritic cell (DC) vaccines, DNA vaccines and peptide/protein vaccines. DC vaccines are generated by pulsing autologous dendritic cells with tumor antigen, which can be either provided as peptides, tumor lysates or tumor cell RNA.

Although NB-specific DC vaccines have been shown to mediate tumor immunity in a NB mouse model, their application in two small phase I clinical trials failed to induce a significant anti-tumor response [43, 44]. NB-specific DNA vaccination approaches focus on survivin [45], tyrosin hydroxylase [46, 47] or MYCN [48] as TAA. Further, DNA vaccines based on GD2 peptide mimotopes are under investigation [49]. These NB-targeting DNA vaccines have been shown to be safe and effective in the induction of antigen-specific T cells, thereby mediating an anti-tumor effect in pre-clinical experiments. Despite these promising pre-clinical results, none of these DNA vaccines has entered a clinical trial so far.

Additionally, protein vaccines based on the application of anti-idiotype antibodies (anti-IdAbs) are under investigation. These anti-IdAbs consist of paratopes that mimic the nominal antigen GD2 and thereby induce the induction of anti-anti-IdAbs, which in turn recognize the nominal antigen on tumor cells. Recently, the generation and characterization of the new anti-IdAb ganglidiomab has been reported [50]. Anti-idiotype properties of ganglidiomab were confirmed *in vitro* and vaccination with ganglidiomab resulted in a GD2-specific anti-NB immune response in a syngeneic NB mouse model. The anti-IdAb 1A7 has been already applied as a GD2 surrogate in a clinical trial with high-risk NB patients [51]. This study revealed the generation of anti-mAb1A7 antibodies in all 31 vaccinated patients and further reported CDC (complement-dependent cytotoxicity) and

ADCC (antibody-dependent cellular cytotoxicity) activity in sera of patients. There were no systemic toxicities and only local reactions to subcutaneous 1A7 application were recorded as adverse events. Vaccination of melanoma patients with the GD2-mimicking anti-IdAb TriGem resulted in a GD2-specific immune response in 40 out of 47 patients [52]. In addition to anti-IdAbs, GD2 peptide mimotopes offer another approach to active immunotherapy in the form of a peptide/protein vaccine. Translation of the weakly immunogenic glycolipid structure of GD2 into the protein structure of GD2 peptide mimotopes provides an important baseline for the induction of a GD2-specific immune response. The application of GD2 peptide mimotopes in a syngeneic mouse model resulted in significantly reduced tumor growth [53].

Although the induction of a long lasting anti-NB immune response by active immunization would be desirable, the immunosuppressed condition of most NB patients after intensive initial therapy remains a major obstacle. Therefore, the most advanced immunotherapeutic anti-NB approaches at the moment are passive immunotherapeutic strategies based on the application of GD2-specific antibodies. The disialoganglioside GD2 is highly expressed on NB cells while physiological expression is restricted, resulting in moderate but manageable side effects of GD2-targeted immunotherapy. Over the last decades, a variety of monoclonal GD2-specific antibodies have been generated and entered clinical trial evaluation. The first antibodies employed in clinical trials were the murine antibodies 3F8 [28, 54, 55] and 14G2a [56-58], which revealed limited tumor responses in some patients, mainly in a minimal residual disease setting, accompanied by generation of human-anti-mouse antibodies (HAMA). Due to their murine nature, 3F8 and 14G2a are immunogenic and thereby induce the generation of those HAMA, resulting in limited anti-tumor activity of the therapeutic murine antibodies. Interestingly, it was shown for some patients that low levels of HAMA antibodies correlated with improved survival [59]. A possible explanation might be provided by the idiotype network theory of Jerne [60]. According to this network theory, some of the generated HAMA antibodies might have anti-idiotype characteristics and specifically bind the paratopes of anti-GD2-antibodies. Hence, these anti-IdAbs mimic the nominal TAA GD2 and can induce GD2-specific antibodies (anti-anti-IdAbs).

Since ADCC in addition to CDC is known to be one of the main mechanisms by which antibodies mediate their anti-tumor effect [61, 62], therapeutic protocols have been adapted to include cytokines, such as IL-2 [63, 64] and granulocyte-macrophage colony-stimulating factor GM-CSF [65] that increase the number of lymphocytes as well as their capacity to mediate ADCC [66, 67].

To reduce the immunogenicity of murine anti-GD2 antibodies, the human/mouse chimeric antibody ch14.18 was generated. This antibody consists of the murine variable regions of 14G2a and human IgG1 constant regions [68]. GD2-binding properties of ch14.18 and 14G2a are comparable and ch14.18 was reported to be even more effective in mediating ADCC towards NB cells [69, 70]. Ch14.18 has been used in several clinical trials, either as a single agent or in combination with cytokines [71-73]. The most promising results of therapeutic application of ch14.18 have been recently shown in a clinical phase III trial conducted by the Childen`s Oncology Group in the US. There, a treatment protocol based on a combination of ch14.18, GM-CSF, IL-2 and 13-cis-retinoic acid resulted in a significant increase of the 2-year event-free survival rate from 46% to 66%. (Fig. 1.3) [74]. The most common side effects of this treatment were pain, hypotension, capillary leak syndrome, fever and hypersensitivity reactions. Pain is thought to be caused by binding of the administered antibody to GD2 on peripheral nerve fibers and subsequent activation of the complement system [75, 76]. The capillary leak syndrome is attributed to systemic IL-2 application [77].

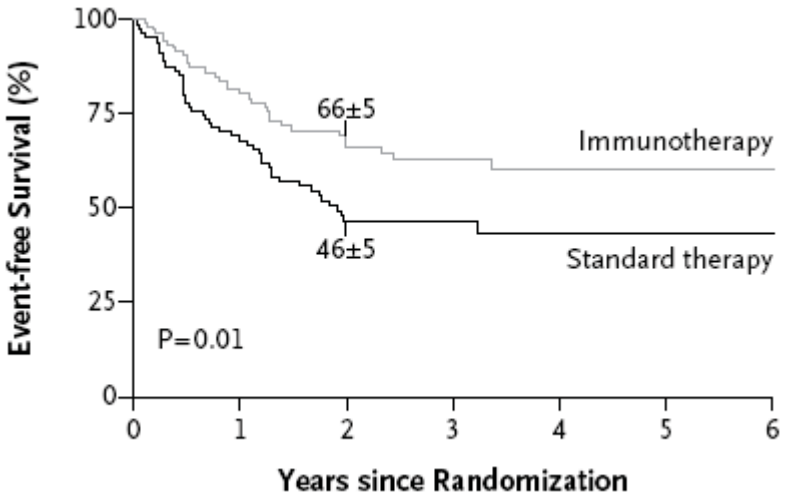


Figure 1.3: Event-free survival of high-risk NB patients [74, modified]. Kaplan-Meier plot for event-free survival of patients either treated with immunotherapy, based on a combination of ch14.18, GM-CSF, IL-2 and 13-cis-retinoic acid or standard therapy. Two year event-free survival rate ± SE is shown for each treatment group.

Since the chimeric antibody ch14.18 still contains murine regions, which can induce the generation of human-anti-chimeric antibodies (HACA), ch14.18 was humanized to further decrease immunogenicity of this anti-GD2 antibody. The humanized antibody hu14.18 is 98% human and contains only the complementarity-determining regions of the original murine antibody. To further increase the potential of GD2-specific antibodies and reduce side effects of a systemic cytokine application, immunocytokines (IC) such as

hu14.18-IL-2 are under current investigation. Immunocytokines were generated to combine the specificity and effector functions of an antibody with the immune stimulating potential of a cytokine, resulting in targeted delivery of the cytokine to the tumor microenvironment. Hu14.18-IL-2 is a fusion protein of the humanized GD2-specific antibody hu14.18 and IL-2 and has already entered clinical trials [78, 79]. These GD2-directed passive immunotherapeutic approaches demonstrated clinical activity and safety to exploit GD2 as TAA in neuroblastoma. This provides an important baseline for further improvement of GD2-directed therapeutic regimens. Along this line, this thesis evaluates a new GD2-directed immunotherapy, based on effector NK cells specifically directed towards GD2 by expression of a chimeric antigen receptor.

1.4. Natural killer cells

Natural killer cells (NK cells) are cells of the innate immune system that mediate lysis of virus-infected or malignant transformed cells and produce immunoregulatory cytokines (IFN-γ, TNF-α and GM-CSF). They are characterized as large granular lymphocytes that lack expression of CD3 [80, 81]. In humans, NK cells can be divided into two subsets with distinct effector functions, according to their CD56 and CD16 expression. The majority of NK cells (90%) characteristically exhibit only low expression of CD56 but high expression of CD16 ($CD56^{dim}$ $CD16^{bright}$), therefore having a cytotoxic phenotype capable of mediating ADCC. In contrast, 10% of all NK cells exhibit high expression of CD56 and low or absent expression of CD16 ($CD56^{bright}$ $CD16^{dim}$). These cells are less cytotoxic but produce immunoregulatory cytokines [82]. In contrast to T cells, NK cells are able to mediate cytotoxicity to target cells without prior sensitization [80, 83].

The complex process of NK cell activation is tightly regulated and based on the interaction of activating or inhibitory receptors, expressed on NK cells, with their respective ligands expressed on target cells. NK cell receptors are germ-line encoded and hence do not have to undergo somatic recombination [84, 85]. Lysis of healthy autologous cells is prevented by signaling through inhibitory receptors, such as KIR (killer cell immunoglobulin-like receptors) or the CD94/NKG2A receptor. Inhibitory KIRs, in contrast to activating KIRs, characteristically express a long cytoplasmic tail including a tyrosine-based inhibitory motif (ITIM), which is also a feature of the inhibitory CD94/NKG2A heterodimer. Inhibitory signaling by KIR and CD94/NKG2A is induced upon engagement of these receptors with allelic variants of HLA class I molecules and HLA-E molecules, respectively [86-88]. Lysis of healthy autologous cells in the absence of activating signaling is thereby prevented. In contrast, decreased or even absent HLA class I expression, which can be

caused by viral infection or malignant transformation, renders cells susceptible to NK cell-mediated lysis (missing self hypothesis) [84, 89].

In addition to these inhibitory receptors, NK cells express a variety of activating receptors. The activating receptor CD16 (FcγRIIIa) is a low affinity receptor for IgG that enables NK cells to mediate ADCC by binding to IgG that specifically recognizes and binds a certain antigen on target cells [90, 91]. Further, activating signaling can be mediated by natural cytotoxicity receptors (NCR), such as NKp46 (NCR1), NKp44 (NCR2) and NKp30 (NCR3) that belong to the Ig-like superfamily. NKp46 and NKp30 are expressed on both resting as well as activated NK cells, in contrast to NKp44, which is only present on activated NK cells [92-94]. Although NCRs have been shown to be involved in NK cell-mediated lysis of different tumor entities, such as NB or leukemia [35, 95], little is known about cellular ligands for NCR. B7-H6 and the HLA-B-associated transcript 3 (BAT3) are known NCR ligands expressed on tumor cells that can mediate NK cell activation through engagement with NKp30 [96, 97]. Additionally, NCRs have been shown to recognize viral components [98-100]. The C-type lectin-like receptor NKG2D is another activating NK cell receptor, which recognizes ligands that are structurally related to MHC class I. This group consists of the MHC class I-related chain (MIC) A and B (MICA and MICB) as well as the unique long 16-binding proteins 1-4 (ULBP1, ULBP2, ULBP3, ULBP4) [101-104]. Expression of these ligands is induced or upregulated on cells upon stress, infection or malignant transformation and has been shown to play a role in cytotoxic activity of NK cells towards melanoma, NB and leukemia cell lines [105]. Activating ligands Nectin-2 (CD112) and the poliovirus receptor (PVR, CD155), which are physiologically expressed on epithelial and endothelial cells and overexpressed on certain tumor entities, can be recognized by Ig-like superfamily member DNAM-1 (dynax-accessory-molecule-1, CD226) [106, 107]. Interaction of DNAM-1 with its ligands has been shown to be involved in NK cell-mediated killing of tumor cells [36, 108, 109].

Due to the wide variety of NK cell receptors expressed, NK cell activation is a very complex process and so far its regulation is not completely understood. It is known though that recognition of IgG by CD16 is sufficient to induce activation by itself, in contrast to other activating receptors, which supposedly have to synergize with each other to induce activation of NK cells [85].

In general, NK cells are capable of inducing apoptosis in target cells by one of two pathways. In the case of predominantly activating signaling, via engagement of activating NK cell receptors with their respective ligands or interaction of CD16 with antibodies that have specifically bound to cellular target antigens, NK cell-mediated induction of apoptosis is based on the release of the pre-stored effector molecules granzyme B and perforin from

cytotoxic granules into the immunological synapse that is formed between NK cell and target cell. Although the exact delivery mechanism of the effector molecules into the target cell is not completely understood, perforin is thought to form a pore within the target cell membrane by polymerization making the cytoplasm of the target cell accessible for granzyme B [110].

The human serine protease family of granzymes consists of granzymes A, B, H, K and M [111]. As one of the most abundant granzymes, granzyme B is one of the major components of cytolytic granules. Inside the target cell granzyme B-mediated apoptosis can be induced by direct activation of the executioner procaspases-3 and -7, resulting in activation of the executioner procaspase-6 and subsequent cleavage of multiple intracellular proteins. Further, granzyme B can directly cleave intracellular housekeeping proteins, such as cytoskeleton components, and is able to translocate into the nucleus to activate the DNA-fragmentation factor (DFF), which induces target cell death. On the other hand, the intrinsic mitochondrial death pathway is an alternative mechanism, by which granzyme B can induce apoptosis in target cells. This is initiated by cleavage of Bid and/or the Mcl-1/Bim complex, resulting in oligomerization of BAX and/or BAK in the outer mitochondrial membrane, which mediates the release of pro-apoptotic proteins such as cytochrome c (cyt-c) into the cytoplasm. Interaction of cyt-c and the apoptotic protease activating factor-1 (Apaf-1) induces formation of the apoptosome that in turn activates pro-caspase-9 [112].

In addition to target cell lysis mediated by ADCC or activating signaling via the above described activating receptors, NK cells can induce apoptosis in target cells via the death receptor pathway. The group of death receptors belongs to the tumor necrosis factor receptor (TNFR) superfamily and consists of six different death receptors [TNFR1 (DR1), Fas (CD95, DR2), DR3, DR4 (TRAIL-R1), DR5 (TRAIL-R2), DR6], all of which express a characteristic death domain (DD) in their cytoplasmic portion. Upon engagement of these death receptors with their ligands (TNF, FasL and TRAIL) expressed on NK cells, oligomerization of receptors and subsequent recruitment of Fas-associated death domain adaptor protein (FADD) and procaspase-8 and -10 result in formation of the death-inducing signaling complex (DISC). Within the context of DISC, procaspase-8 and -10 are cleaved into their active form and released into the cytoplasm. Caspase-8 and -10 promote apoptosis either via direct activation of executioner caspases-3 and -7 or induction of the mitochondrial death pathway [113, 114]. Based on the variety of pathways by which NK cells can be activated and subsequently lyse malignant cells, NK cells are an attractive effector cell population for immunotherapy. Since functionality or NK cell counts can be negatively affected in cancer patients, NK cell lines are an alternative NK cell source. The

GD2-directed immunotherapeutic approach evaluated in the present study is based on CAR-mediated redirection and adoptive transfer of the human NK cell line NK-92.

1.5. NK-92

The human NK cell line NK-92 was generated from a 50-year-old patient with Non-Hodgkin's lymphoma and exhibits phenotypical and functional characteristics of activated NK cells [115]. NK-92 cells have been shown to mediate high cytotoxicity towards a variety of tumor cell lines as well as primary tumor cells [116-118]. Characterization of surface marker expression revealed expression of $CD56^{bright}$, CD2, CD7, CD11a, CD28, CD45 and CD54, whereas expression of CD1, CD3, CD4, CD5, CD8, CD10, CD14, CD16, CD19, CD20, CD23, CD34 and HLA-DR could not be detected. Survival and growth of NK-92 are IL-2 dependent [116]. Due to lack of expression of the low affinity IgG Fc receptors CD16 and CD32 as well as the high IgG affinity receptor CD64, NK-92 are not capable of mediating ADCC by themselves [119]. Regarding the expression of activating NK cell receptors, it has been shown that NK-92 express NKp30, NKp46, 2B4 and NKG2D. Further, NK-92 cells express TRAIL, TNF-α and low levels of FasL, indicating that they are able to mediate cytotoxicity using the death receptor/death receptor ligand pathway [119]. Expression of the majority of inhibitory NK cell receptors in particular of KIRs is absent on NK-92. The only exceptions are KIR2DL4, which is an unusual KIR and has been shown to mediate inhibitory as well as activating signaling [120] and the CD94/NKG2A heterodimer [119]. Importantly, despite the lack of inhibitory receptors, NK-92 do not mediate cytotoxicity towards non-malignant allogeneic cells or negatively affect the normal proliferative potential of hematopoietic cells [116].

All the above mentioned characteristics, and the fact that NK-92 can be easily expanded under good manufacturing practice conditions (GMP) [121, 122], support NK-92 as an interesting effector cell line for an immunotherapeutic approach. Regarding the safety of an NK-92 application, irradiation of NK-92 cells with 500 cGy was found to prevent cell proliferation while retaining cytotoxic activity [123]. So far, safety and tolerability of an application of irradiated NK-92 in patients has been shown in a clinical phase I trial [124]. Recently, the application of NK-92 in a phase I trial demonstrated persistence of NK-92 for up to 48 hours after infusion and showed some responses in patients with advanced lung cancer [125]. To address the problem of NK cell resistance due to reduced expression or release of activating NK cell ligands on tumor cells, and to specifically direct this highly cytotoxic NK cell line towards distinct tumor entities, NK-92 can be genetically engineered to express TAA-specific chimeric antigen receptors.

1.6. Chimeric antigen receptors

The concept of chimeric antigen receptors (CARs) was developed to combine two existing immunotherapeutic approaches, the targeting of a specific antigen with an antibody and the adoptive transfer of immune effector cells. This is based on the idea that a combination of the specificity of an antibody and the ability of effector cells to penetrate tissues and to directly mediate cytotoxicity towards target cells would further improve therapeutic potential. The first chimeric antigen receptors were designed as a combination of variable regions of the heavy and the light chain (V_H and V_L) of an antibody and the extracellular constant regions as well as the transmembrane and cytoplasmic domains of a T cell receptor (TCR) [126]. Although these chimeric T cell receptors had been shown to be functional, further improvements were made to create CARs, consisting of single chain fragments (scFv) for antigen recognition, which are connected via a hinge region to signal-transducing receptor subunits, such as the CD3 ζ-chain or the FcγRIII γ-chain. Single chain fragments contain the variable regions of the heavy (V_H) and the light chain (V_L) of an antibody, connected by a flexible linker [127, 128]. Specificity and affinity of such single chain fragments have been shown to be comparable to the Fab fragment of the respective antibody [129].

Further improvements had been made to these first generation CARs, resulting in second generation CARs which include an additional costimulatory signaling domain such as CD28, CD134 (OX40) or CD137 (4-1BB), and third generation CARs that contain a combination of several additional costimulatory signaling domains (Fig. 1.6) [130-132]. Inclusion of these additional costimulatory domains could increase cytotoxic activity and cytokine production, as well as trafficking and persistence of CAR-expressing effector cells [132-134].

Different methods have been employed for genetic engineering of effector cells. Permanent expression of the CAR-transgene in effector cells can be achieved by the use of retroviral or lentiviral vectors [135, 136]. Additionally, approaches based on electroporation to transfect effector cells with mRNA were successful in inducing transient expression of the CAR-transgene [137].

The concept of chimeric antigen receptors has great potential for cancer immunotherapy, since expression of CARs especially on T cells enables these effector cells to specifically target a broader range of surface TAA in addition to proteins, including sugars or lipids, which they could otherwise not recognize by their conventional MHC-restricted mechanism of antigen recognition. This is particularly important with regard to the down-regulated/decreased MHC class I expression by which tumor cells can escape immune recognition.

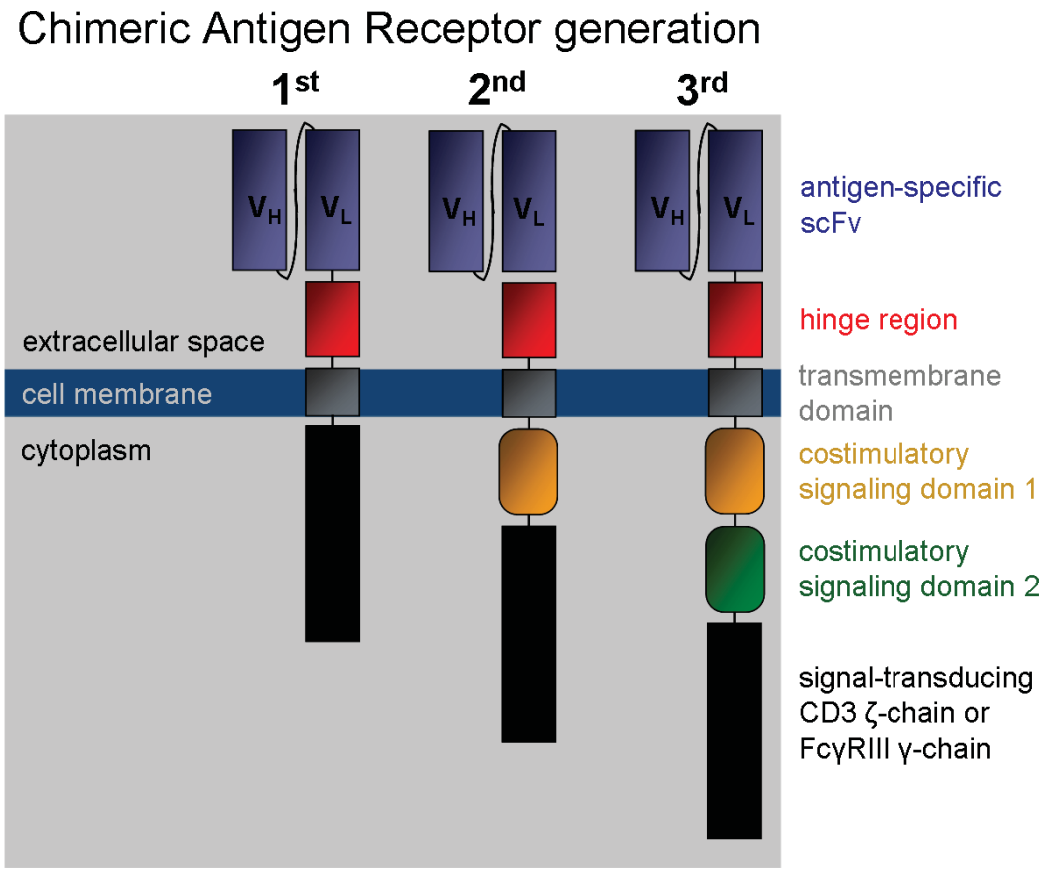


Figure 1.6: Chimeric antigen receptor (CAR) generations (adapted from [138]). First generation CARs consist of an antigen-specific single chain fragment variable (blue), a hinge region (red) to separate the scFv from the transmembrane domain, a transmembrane domain (gray), and an intracellular signal-transducing domain, such as the CD3 ζ-chain or the FcγRIII γ-chain (black). Second generation and third generation CARs additionally include one or multiple costimulatory signaling domains (yellow, green), such as CD28, CD134 (OX40) or CD137 (4-1BB), respectively.

In addition to T cells, NK cells are promising effector cells for CAR-based immunotherapeutic approaches. MHC class I downregulation, such as in the case of NB, already renders tumors susceptible to NK cell-mediated cytotoxicity due to lack of inhibitory signals mediated in healthy tissues by the interaction of MHC class I molecules and inhibitory KIR receptors on NK cells. Since activating NK cell receptors are germline encoded, recognition of target cells is restricted to a limited number of conserved activating ligands on tumor cells. Hence, genetic engineering of NK cells to express a CAR will highly increase specificity of NK cells to a broad range of surface TAA on tumor cells. This has the potential to address the problem of immune evasion by down-regulation or shedding of activating ligands on tumor cells.

So far, CAR-expressing effector cells have been generated to specifically target different TAAs, such as ErbB2, CD20, CD19 and GD2 [139-144]. Importantly, CAR-

expressing effector cells have already entered clinical trials and have shown some promising results [145-147]. In the present study, CAR technology was employed to expand passive immunotherapy directed against GD2 as NB-associated target antigen to a cellular approach based on NK cells as effector cells. In particular, NK-92 were genetically modified to express a GD2-specific CAR (NK-92-scFv(ch14.18)-zeta.

1.7. NK-92-scFv(ch14.18)-zeta

The concept of genetic engineering of immune effector cells to express a CAR was employed to combine passive immunotherapeutic approaches based on TAA-specific antibodies with the application of the human NK cell line NK-92, which has been demonstrated to be safe and well tolerated, with some responses in patients [122, 124, 125]. So far, CAR-expressing NK-92 cell lines have been generated towards a variety of TAAs, such as CD20 or CD19 in lymphoma and leukemia cells and ErbB2 or EpCAM in tumors of epithelial origin. The efficacy of these cell lines has been demonstrated in pre-clinical *in vitro* and *in vivo* studies [140, 142, 148, 149].

Since GD2-specific passive immunotherapeutic approaches based on the application of the GD2-specific antibody ch14.18 have been shown to significantly improve survival of high-risk NB patients [74], the GD2-specific NK cell line NK-92-scFv(ch14.18)-zeta was generated to extend the approach of CAR-expressing NK-92 to an application in NB.

NK-92-scFv(ch14.18)-zeta was previously generated by genetic engineering of parental NK-92 cells to express a GD2-specific chimeric antigen receptor [150]. GD2-specificity of this CAR is mediated by a single chain fragment (scFv(ch14.18)), containing the variable regions of the heavy (V_H) and the light chain (V_L) of the GD2-specific antibody ch14.18, connected by a synthetic flexible linker ($(G_4S)_4$). A CD8α hinge region connects the scFv to an extracellular Myc-tag, which is included to enable detection of the expressed CAR on the surface of transduced cells.

Signal-transduction is mediated by the CD3 ζ-chain, which is linked to the Myc-tag. Further, the neomycin-resistance gene is included in the vector to enable selection of successfully transduced cells by addition of G418 in the culture media [150]. Positions of all components within the retroviral vector pLXSN are shown in Figure 1.7.

Since it was unknown, whether orientation of V_H and V_L might affect functionality of the resulting CAR, vectors with both orientations were used for the generation of NK-92-scFv(ch14.18)-zeta as described previously [150]. No differences in expression of CARs with both orientations were observed. Hence, we received the clone with the V_HV_L orientation from the collaborating lab to perform the experiments presented in this study.

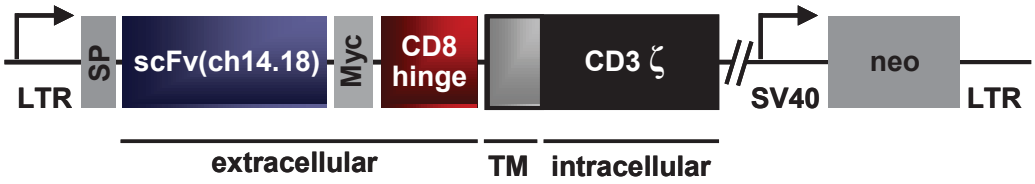


Figure 1.7: Retroviral vector encoding for GD2-specific CAR (scFv(ch14.18)-zeta) [150, modified]. Expression of scFv(ch14.18)-zeta is controlled by a 5'LTR (long terminal repeat; from moloney murine leukemia virus). N-terminal immunoglobulin heavy chain leader peptide SP (signal peptide) is followed by the single chain fragment containing the variable regions of the heavy and the light chain of ch14.18 (scFv(ch14.18), a Myc-tag, CD8α hinge region and the CD3 ζ-chain. Additionally, the SV40 promotor drives the expression of a neomycin resistance gene, which was included for selection of transduced cells with G418.

In theory, these newly generated NK-92-scFv(ch14.18)-zeta should be able to mediate cytotoxicity towards tumor cells by either one or a combination of three different ways in the absence of inhibitory signaling due to lack of inhibitory receptors: (1) activation by interaction of activating NK receptors with respective ligands on tumor cells, (2) activation by engagement of the CAR with the TAA GD2, both of which result in the release of the effector molecules granzyme B and perforin, and (3) induction of apoptosis in tumor cells by interaction of death receptor ligands on NK cells with death receptors expressed on tumor cells.

1.8. Aim of this study

The treatment of high-risk NB is particularly challenging due to the development of drug-resistance during induction and consolidation therapy. Drug resistance associated with progressive disease and in particular MRD, which can cause a relapse, is a major challenge in the treatment of high-risk NB with conventional therapies. Although passive immunotherapeutic approaches targeting the TAA GD2 have recently shown promising results, there is still need for the development of new and more effective treatment strategies to further improve survival of high-risk patients.

Therefore, this study aims at analyzing the mechanism and the efficacy of a GD2-specific cellular therapy with NK cells. For this purpose, we employed NK-92-scFv(ch14.18)-zeta cells. Expression of the GD2-specific CAR should enable NK-92-scFv(ch14.18)-zeta to overcome evasion mechanisms developed by the tumor. Within this study we wanted to evaluate in detail, GD2-specificity, and the role of the interaction between CAR and GD2 in both activation of NK-92-scFv(ch14.18)-zeta and lysis mediated by NK-92-scFv(ch14.18)-zeta. Additionally, *in vivo* therapeutic efficacy of NK-92-scFv(ch14.18)-zeta was determined in a drug-resistant xenograft mouse model.

2. Material and Methods

2.1. Material

2.1.1. Chemicals and supplements

Product		Provider
Acrylamide Bisphosphate		BioRad, Munich, Germany
Ammoniumpersulfate		BioRad, Munich, Germany
Bovine serum albumin	(BSA)	Sigma-Aldrich, Steinheim, Germany
Calcein-AM		Sigma-Aldrich, Steinheim, Germany
^{51}Chromium		PerkinElmer, Billerica, MA, USA
Diamino-phenylindole	(DAPI)	Sigma-Aldrich, Steinheim, Germany
Dimethylsulfoxide	(DMSO)	WAK Chemie Medical GmbH, Steinbach, Germany
Ethanol ≥ 99.8%		Carl Roth GmbH, Karlsruhe, Germany
Ethylenediaminetetraacetic acid	(EDTA)	Carl Roth GmbH, Karlsruhe, Germany
GM2		Sigma-Aldrich, Steinheim, Germany
GD2	(disialoganglioside)	Sigma-Aldrich, Steinheim, Germany
Human albumin		CSL Behring GmbH, Marburg, Germany
Ionomycin		Sigma-Aldrich, Steinheim, Germany
Methanol ≥ 99.9%		Carl Roth GmbH, Karlsruhe, Germany
2-mercaptoethanol		Sigma-Aldrich, Steinheim, Germany
Phosphate buffered saline w/o Ca^{2+} and Mg^{2+}	(PBS)	PAA, Pasching, Austria
Phorbol myristate acetate	PMA	Calbiochem, EMD Biosciences Inc., La Jolla, CA, USA

1-Phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol	(PPPP)	Dr. Barry Maurer, Texas Tech University, Health Sciences Center Cancer Center, Lubbock, TX, USA
2-Propanol		Carl Roth GmbH, Karlsruhe, Germany
Propidiumiodide	(PI)	Molecular Probes, Life Technologies, Darmstadt, Germany
Sodium azide	NaN_3	Carl Roth GmbH, Karlsruhe, Germany
Sodium chloride		Merck KGaA, Darmstadt, Germany
Sodium dodecyl sulphate	(SDS)	BioRad, Munich, Germany
Sulphuric acid	(H_2SO_4)	Carl Roth GmbH, Karlsruhe, Germany
N,N,N`,N`tetra-methyl-ethylendiamine	(TEMED)	BioRad, Munich, Germany
Tris-HCl		BioRad, Munich, Germany
Triton-X-100		Carl Roth GmbH, Karlsruhe, Germany
Tween-20		BioRad, Munich, Germany

2.1.2. Cell culture media und supplements

Cell culture medium	**Provider**
IMDM	PAA, Pasching, Austria
RPMI 1640	PAA, Pasching, Austria
X-VIVO-10	Biozym Scientific GmbH, Hessisch Oldendorf, Germany

Supplement		**Provider**
Fetal bovine serum	(FBS)	PAN-Biotech, Aidenbach, Germany
Geneticin	(G418)	Sigma-Aldrich, Steinheim, Germany
Human serum AB	converted	PAA, Pasching, Austria
Interleukin-2	(Aldesleukin, $18x10^6$ IU/ml)	Novartis, Nuremberg, Germany

ITS (1000x)	(ITS, 5 mg/ml insulin, 5 mg/ml transferrin, 5 µg/ml selenious acid)	BD Biosciences, Heidelberg, Germany
Penicillin/Streptomycin	10,000 IU/ml / 10 mg/ml	PAA, Pasching, Austria
Stable glutamine	200 mM	PAA, Pasching, Austria

2.1.3. Special laboratory reagents and buffers

Product	Provider
Trypan blue 0.4%	Sigma-Aldrich, Steinheim, Germany
0.05% Trypsin/EDTA	PAA, Pasching, Austria
Protease inhibitor cocktail	Sigma-Aldrich, Steinheim, Germany
BioRad protein assay	BIO-RAD, Munich, Germany
Laemmli sample buffer	BioRad, Munich, Germany
Spectra™ Multicolor Broad Range Protein Ladder	Thermo Scientific, Erlangen, Germany
Matrigel™ Basement Membrane Matrix	BD Biosciences, Heidelberg, Germany
Immun-Star- HRP peroxide buffer	BioRad, Munich, Germany
Immun-Star-HRP luminol/enhancer	BioRad, Munich, Germany

2.1.4. Kits

Human granzyme B ELISA Kit	Cat# 3485-1H-20	MabTech, Hamburg, Germany
Human perforin ELISA Kit	Cat# 3465-1H-20	MabTech, Hamburg, Germany

2.1.5. Antibodies

Primary antibodies

antigen	clone/name	isotype	conjugate	Cat #	provider
GD2	ch14.18/CHO	human IgG1	purified	/	Polymun, Vienna, Austria
14G2a	Id14G2a-17-9 (anti-IdAb, ganglidiomab)	mouse IgG1, κ	purified	/	BioGenes, Berlin, Germany
Myc-tag	9E10	mouse IgG1, κ	purified	SAB4700447	Sigma-Aldrich, Steinheim, Germany
GCS	1E5	mouse IgG1, κ	purified	H00007357-M03	Abnova, Heidelberg, Germany
β-actin	AC-15	mouse IgG1, κ	HRP	A3854	Sigma-Aldrich, Steinheim, Germany

Isotype controls

isotype	clone/name	conjugate	Cat #	provider
human IgG1	Rituximab/MabThera	purified	/	Roche, Mannheim, Germany
mouse IgG1	11711	purified	MAB002	R&D systems, Wiesbaden-Nordenstadt, Germany

Secondary antibodies

specificity	clone/name	isotype	conjugate	Cat #	provider
human-IgG	G18-145	Mouse IgG1	PE	555787	BD Biosciences
mouse IgG1	A85-1	Rat IgG1	PE	550083	BD Biosciences
mouse IgG1	/	goat	HRP	170-6516	BioRad, Munich, Germany

2.1.6. Cell culture media and buffers preparations

Cell culture media	
RPMI 1640	
10%	FBS
100 IU/ml	Penicillin
0.1 mg/ml	Streptomycin
IMDM	
20%	FBS
1 x	ITS (5 µg/ml Insulin, 5 µg/ml transferrin, 5 ng/ml selenious acid)
4 mM	stable glutamine
X-VIVO-10	
5%	human serum, type AB, *converted*
100 IU/ml	IL-2 (Aldesleukin)
0.6 mg/ml	G418 (Geneticin)
Western blot buffers	
Resolving gel buffer pH 8,8	
1.5 M	Tris-HCl
0.4%	SDS
Stacking gel buffer pH 6.8	
0.5 M	Tris-HCl
0.4%	SDS

Running buffer	
25 mM	Tris
192 mM	Glycin
0.1%	SDS
Washing buffer (TTBS) pH 7.5	
20 mM	Tris
150 mM	NaCl
0.05%	Tween 20
Blocking buffer	
	PBS
1x	Roti-Block
Transfer buffer (Towbin buffer) pH 8.3	
25 mM	Tris
192 mM	Glycin
5%	Methanol
Lysis buffer	
10 mM	Tris
10 mM	NaCl
0.1 mM	EDTA
0.5%	Triton-X 100
0.02%	NaN_3

FACS buffer	
	PBS
1%	BSA
0.1%	EDTA
0.1%	NaN_3

ELISA buffers	
Washing buffer	
	PBS
0.05%	TWEEN 20

Assay diluent	
	PBS
0.1%	BSA

2.1.7. Special laboratory tools

Protran nitrocellulose transfer membrane	Whatman GmbH, Dassel, Germany
Mini trans-blot filter paper	BioRad, Munich, Germany
70 µm cell strainer	BD Biosciences, Heidelberg, Germany

2.1.8. Special laboratory equipment

Synergy HT multi-mode microplate reader	BioTek, Bad Friedrichshall, Germany
FACS Canto II	BD Biosciences, Heidelberg, Germany
Wizard 2 Gamma counter	PerkinElmer, Billerica, MA, USA

Mini Trans-Blot® Electrophoretic Transfer Cell	BioRad, Munich, Germany
Mini-PROTEAN® cell	BioRad, Munich, Germany
ChemiDoc™ XRS+ Molecular Imager	BioRad, Munich, Germany

2.1.9. Softwares

FlowJo 7.6	Treestar, Ashland,OR
FACSDiva 6.1.3	BD Biosciences, Heidelberg, Germany
GraphPad Prism 5.01	GraphPad software, San Diego, CA
Sigma Plot 10.0	Systat Software GmbH, Erkrath, Germany

2.1.10. Cell lines

All NB cell lines (LA-N-1, LA-N-5, SK-N-BE(1), SK-N-BE(2), CHLA-15, CHLA-20, CHLA-79, CHLA-136, SMS-KAN, SMS-KANR, SMS-KCN, SMS-KCNR, SK-N-SH) and the erythroleukemia cell line K562 were kindly provided by Dr. Reynolds' laboratory (Texas Tech University, Health Sciences Center Cancer Center, Lubbock, TX, USA), which houses the Children's Oncology Group (COG) cell line repository. Some of the NB cell lines used were generated from the same patient at different points in therapy and are referred to as cell line pairs. The cell lines SK-N-BE(1), SMS-KAN, SMS-KCN and CHLA-15 were established at time point of diagnosis. SK-N-BE(2), SMS-KANR, SMS-KCNR and CHLA-20 were generated from relapse tumor material of the same patient, respectively.
Drug resistance patterns of the above mentioned NB cell lines had been analyzed in previous studies. These reported drug resistance of CHLA-20 and multidrug resistance of SK-N-BE(2), CHLA-79 and CHLA-136 [16, 151, 152].

The parental human NK cell line NK-92, as well as the empty vector control cell line NK-92-pLXSN, ErbB2-specific NK-92-scFv(FRP5)-zeta [140] and GD2-specific NK-92-scFv(ch14.18)-zeta [150], were kindly provided by Prof. Wels (Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt, Germany).

2.2. Methods

2.2.1. Cell culture

All cell lines were cultured under standard conditions (37°C, 100% relative humidity, 5% CO_2, 95% air). The human NB cell lines SK-N-BE(1), SK-N-BE(2), SMS-KAN, SMS-KANR, SMS-KCN, SMS-KCNR, LA-N-1, LA-N-5, SK-N-SH as well as the human erythroleukemia cell line K562 were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 IU/ml Penicillin and 0.1 mg/ml Streptomycin. CHLA-15, CHLA-20, CHLA-79 and CHLA-136 were cultured in IMDM supplemented with 20% heat-inactivated FBS, 4 mM glutamine, 1x ITS, 100 IU/ml Penicillin and 0.1 mg/ml Streptomycin.

The parental human NK cell line NK-92 was cultured in X-VIVO-10 supplemented with 5% heat-inactivated human AB serum and 100 IU/ml human recombinant IL-2. X-VIVO-10 containing 5% heat-inactivated human AB serum, 100 IU/ml human recombinant IL-2 and 0.6 mg/ml G418 was used to culture NK-92-pLXSN, NK-92-scFv(FRP5)-zeta as well as NK-92-scFv(ch14.18)-zeta.

Adherent cells were subcultured at 80-90% confluency using 0.05% Trypsin/EDTA. For cryoconservation, NB cells and K562 were frozen in human albumin containing 10% DMSO at a concentration of 3-6x10^6 cells/ml. NK cells were frozen in X-VIVO-10 containing 5% human AB serum, 100 IU/ml IL-2 and 10% DMSO at a concentration of 5x10^6 cells/ml. For optimal viability, cells were frozen at -80°C for 72 h at a cooling rate of -1°C to -3°C/min using an isopropanol chamber. After 72 h, cells were transferred to a liquid nitrogen tank for long-term storage. Identities of all NB cell lines, K562 and NK-92 were verified by analysis of short tandem repeats (STR), as described in the literature [153], and verified against the COG cell line STR database. Further, all cell lines were routinely tested for mycoplasma contamination using the MycoAlert-Assay from LONZA (Walkersville, MD, USA).

2.2.2. PPPP-treatment of NB cell lines

To determine the impact of GD2 recognition on NK-92-scFv(ch14.18)-zeta-mediated lysis of NB tumor cells, GD2-expression was inhibited by pre-treating cells with the selective glucolysceramide synthase (GCS) inhibitor PPPP (1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol, kindly provided by Dr. Barry Maurer, Texas Tech University, Health Sciences Center Cancer Center, Lubbock, TX, USA, [154]). GCS catalyzes the conversion of ceramides into glucosylceramides, which is the first step in GD2-synthesis. For PPPP-treatment NB cells (CHLA-20 and SK-N-BE(2)) were seeded into a 6-well plate at a concentration of 1x10^6 cells in 3 ml/well and allowed to attach

overnight. PPPP was added in a final concentration of 1 µM. Control cells were treated with the same volume of 100% EtOH (vehicle control). Cells were incubated with PPPP or vehicle control for three days. GD2-expression was confirmed using flow cytometry and sensitivity towards NK-92-scFv(ch14.18)-zeta-mediated lysis was analyzed in ^{51}Cr release cytotoxicity assays as described below.

2.2.3. Flow cytometry

Exclusion of dead cells in flow cytometric analyses was done by addition of PI or DAPI prior to sample acquisition, at a concentration of 1 mg/ml and 0.1 mg/ml, respectively. Flow cytometric analyses were performed at a BD FACS CANTOII using FACSDiva software (BD Biosciences, Heidelberg, Germany), and data was analyzed using FlowJo (Treestar, Ashland, OR, USA).

2.2.3.1. Flow cytometric analysis of CAR-expression

CAR surface expression can be analyzed with two different staining strategies. For analysis based on Myc-tag-expression within the extracellular part of the CAR, cells were incubated with anti-Myc-tag antibody (1 µg/1x10^6 cells, diluted in FACS buffer) in a total volume of 100 µl for 30 min on ice in the dark. For analysis of GD2-specific CAR-expression, NK cells were stained with the anti-idiotype antibody (anti-IdAb) ganglidiomab [50] (Id14G2A-17-9, 1 µg/1x10^6 cells) in a total volume of 100 µl for 30 min on ice in the dark. Due to its anti-idiotype characteristics, ganglidiomab specifically binds the paratopes of the GD2-specific CAR. Purified mouse IgG1 (1 µg/1x10^6 cells) was utilized as isotype control. Cells were washed with 1 ml FACS buffer and centrifuged (300x g, 5 min, RT). Supernatant was discarded, and cells were incubated with PE-labeled rat anti-mouse IgG1 antibody (1:200) in a total volume of 100 µl for 20 min on ice in the dark. Cells were washed with 1 ml of FACS buffer and centrifuged (300x g, 5 min, RT). Supernatant was discarded; cells were resuspended in 500 µl FACS buffer and transferred into a FACS tube. For each sample, 20,000 live cells were analyzed.

To analyze the effect of IL-2 starvation on CAR expression, cells were cultured for four days in X-VIVO-10 with 5% human serum and 0.6 mg/ml G418 (IL-2 starvation) or in X-VIVO-10 containing 5% human serum, 0.6 mg/ml G418 and 100 IU/ml IL-2 (complete). Starvation was abrogated after four days by addition of IL-2 and culture of cells for two additional days.

2.2.3.2. Flow cytometric analysis of GD2-expression

For analysis of GD2 surface expression, cells were stained with ch14.18/CHO (1 µg/1x10^6 cells, diluted in FACS buffer) in a total volume of 100 µl for 20 min on ice in the

dark. The chimeric anti-CD20 antibody rituximab (1 µg/1x10^6 cells) was used as isotype control for ch14.18. Cells were washed with 1 ml FACS buffer and centrifuged (300x g, 5 min, RT). Supernatant was discarded and cells were incubated with PE-labeled mouse anti-human IgG antibody (1:5, diluted in FACS buffer) in a volume of 100 µl for 20 min on ice in the dark. Cells were washed with 1 ml FACS buffer, centrifuged (300x g, 5 min, RT) and supernatant was discarded. Cells were then resuspended in 500 µl FACS buffer and transferred to a FACS tube for flow cytometric analysis. Ch14.18/CHO was kindly provided by the SIOPEN (International Society of Pediatric Oncology Europe Neuroblastoma) group. For each sample, 20,000 live cells were analyzed.

2.2.4. Cytotoxicity assays

Two different kinds of cytotoxicity assays were employed to analyze the cytotoxic activity of NK-92-scFv(ch14.18)-zeta as well as control NK cell lines. The ^{51}Cr release assay is a radioactive cytotoxicity assay based on the labeling of target cells with ^{51}Cr prior to co-incubation with effector cells. Killing of target cells then results in release of radioactive ^{51}Cr that can be detected in the supernatant with a gamma counter. Alternatively, the calcein release cytotoxicity assay is a non-radioactive cytotoxicity assay and based on the labeling of target cells with calcein-acetoxymethylester (calcein-AM). Within the cell, calcein-AM is converted by intracellular esterases to calcein, which cannot passively cross the intact cell membrane. Effector cell-mediated cell killing results in release of calcein from dead cells into the supernatant. Calcein is a fluorescent dye with an excitation maximum at a wavelength of 495 nm and an emission maximum at 515 nm. Fluorescence of calcein in the supernatant can be measured with a microplate reader (filters: 485/20 and 528/20).

2.2.4.1. ^{51}Cr release cytotoxicity assay

^{51}Cr release cytotoxicity assay was used to analyze the cytotoxicity of NK-92-scFv(ch14.18)-zeta and parental NK-92 or empty vector control NK-92-pLXSN towards GD2-positive cell lines (CHLA-136, CHLA-79, CHLA-20, SK-N-BE(2), LA-N-1, LA-N-5). Further, sensitivity of NB cell line pairs of four patients (SK-N-BE(1)/SK-N-BE(2), SMS-KAN/SMS-KANR, SMS-KCN/SMS-KCNR, CHLA-15/CHLA-20) towards NK-92-scFv(ch14.18)-zeta and control NK cell line-mediated cytotoxicity was compared. ^{51}Cr release assays were also performed to determine effect of inhibition of GCS and thereby GD2-expression on sensitivity of PPPP or vehicle pre-treated CHLA-20 and SK-N-BE(2) target cells towards NK-92-scFv(ch14-18)-zeta and control NK cell line-mediated cytotoxicity.

Target cells were loaded with ^{51}Cr (0.125 mCi/5x10^5 cells in 500 µl) and incubated for two hours at 37°C. Cells were washed twice with X-VIVO-10 containing 5% heat-inactivated human AB serum and 5x10^3 target cells were then co-incubated with effector cells at an E:T ratio of 6.3:1 in a final volume of 200 µl. Maximum release was induced by addition of 5% SDS solution to target cell suspension. To assess for spontaneous ^{51}Cr release 5x10^3 target cells were incubated without NK cells. After incubation for six hours, 50 µl of supernatant were transferred into a test tube, and radioactivity was measured using a gamma counter Wizard 2 (PerkinElmer, Billerica, MA, USA). Specific cytotoxicity was calculated according to the formula:
((experimental lysis – spontaneous lysis) / (maximal lysis – spontaneous lysis)) x 100.

To block CAR-mediated lysis, 10 µg/ml anti-IdAb ganglidiomab were added during co-incubation of NK-92-scFv(ch14.18)-zeta with either LA-N-5 or LA-N-1. Purified mouse IgG1 (10 µg/ml) was used as isotype control.

2.2.4.2 Calcein release cytotoxicity assay

The calcein release cytotoxicity assay was used to determine an appropriate E:T ratio in assays with CHLA-20 as target cells. Further, the cytotoxic activity of NK-92-scFv(ch14.18)-zeta and control cell lines towards $GD2^+$ CHLA-20 and $GD2^-$ SK-N-SH was analyzed using calcein release assays. The effect of CAR-blocking as well as the effect of blocking the target antigen GD2 on NK-92-scFv(ch14.18)-zeta-mediated lysis of $GD2^+$ CHLA-15 and CHLA-20 in comparison to $GD2^-$ K562 were further analyzed with this assay.

For calcein labeling 6x10^5 target cells were resuspended in 1 ml PBS/12.5% FBS, and calcein-AM was added at a final concentration of 10 µM. Cells were incubated shaking (100 rpm) for 30 min at 37°C, without CO_2 and then washed twice with PBS/12.5% FBS. Next, cells were washed once in X-VIVO-10 containing 5% heat-inactivated human AB serum, centrifuged (300x g, 5 min, RT). Target cells were then resuspended in 12 ml X-VIVO-10/5% heat-inactivated human serum for a final concentration of 5x10^3 cells/100 µl per well. Effector cells were washed once in X-VIVO-10/5% heat-inactivated human serum and resuspended in X-VIVO-10/5% heat-inactivated human serum at a final concentration of 3.15x10^4 cells/100 µl/well for an E:T ratio of 6.3:1. For maximal calcein release, target cell suspension was diluted 1:2 in X-VIVO-10/5% human serum, treated with ultrasound for 30 s and then 200 µl/well were added to respective wells. After incubation for five hours at 37°C without CO_2, 96-well plates were centrifuged (300x g, 5 min, RT) and 50 µl of supernatant were transferred into a black 96-well plate. Calcein fluorescence in supernatants was then analyzed at an excitation wavelength of 495 nm and emission wavelength of 515 nm using a microplate reader. Specific cytotoxicity was calculated

according to the formula: ((experimental lysis – spontaneous lysis) / (maximal lysis – spontaneous lysis)) x 100.

Blocking of the CAR during co-incubation of target cells and NK-92-scFv(ch14.18)-zeta was accomplished by addition of 10 µg/ml anti-IdAb ganglidiomab or mouse IgG1 as the isotype control.

The target antigen GD2 was blocked by addition of the GD2-specific antibody ch14.18/CHO during co-incubation with effector cells at a final concentration of 10 µg/ml. Chimeric CD20-specific antibody rituximab (10 µg/ml) was used as the isotype control.

2.2.5. Granzyme B and Perforin ELISA

Granzyme B and perforin production of NK-92-scFv(ch14.18)-zeta and NK-92-pLXSN in response to activation with immobilized GD2 were analyzed with ELISA. Therefore, NK cells were incubated with 100 ng plate-bound GD2. The same amounts of GM2 were used as an unspecific control. Immobilization of gangliosides was achieved by incubation of 100 ng GD2 (100 µl, 1 ng/µl) or GM2 (100 µl, 1 ng/µl) in 100% methanol/well in a 24-well plate for one hour at 56°C to evaporate the methanol. $5x10^5$ NK-92-scFv(ch14.18)-zeta or NK-92-pLXSN were added in a total volume of 500 µl X-VIVO-10 containing 5% heat-inactivated human serum, and incubated for six hours at 37°C. As a positive control, NK cells were stimulated with 10 ng/ml PMA and 1 µg/ml Ionomycin.

To block GD2-induced granzyme B and perforin production, the anti-IdAb ganglidiomab was added at a final concentration of 10 µg/ml during incubation. Purified mouse IgG1 was utilized as an isotype control for ganglidiomab. After six hours, 500 µl of supernatant were collected, centrifuged (300x g, 5 min, RT) and stored at -80°C until further analysis with ELISA.

Granzyme B and perforin concentrations were determined using granzyme B and perforin ELISA kits. For this purpose, a 96-well ELISA plate was coated with either monoclonal anti-granzyme B antibody (GB10, 150 ng/well in 100 µl) or monoclonal anti-perforin antibody (Pf-80/164, 200 ng/well in 100 µl) and incubated overnight at 4°C. The next day, plates were washed twice with washing buffer and incubated with 200 µl/well assay diluent for one hour at room temperature to block unspecific binding. After washing plates three times with washing buffer, 100 µl of standard or sample were added into respective wells. The granzyme B standard was 1:2 serially diluted with concentrations ranging from 10.3 pg/ml to 1,300 pg/ml, and the perforin standard 1:2 dilutions ranged from 31.25 pg/ml to 4,000 pg/ml. Plates were incubated at room temperature for two hours and washed three times with washing buffer. For granzyme B and perforin detection, 100 µl of 0.5 µg/ml GB11-biotin and 1 µg/ml Pf-344-biotin, respectively, were added to each well and incubated at room temperature for one hour. After plates were washed three times with

washing buffer, 100 µl/well Streptavidin-HRP (1:1,000 in assay diluent) were added for one hour at room temperature. Plates were washed three times with washing buffer and 75 µl of TMB substrate solution were added to each well. The reaction was stopped using 75 µl/well 2N H_2SO_4, and samples were then analyzed with an ELISA reader at 450 nm.

2.2.6. Western Blot for glucosylceramide synthase (GCS)

For analysis of GCS-expression using Western blot, cell pellets of $5x10^6$-$1x10^7$ cells were collected from SK-N-BE(1)/SK-N-BE(2), SMS-KAN/SMS-KANR, SMS-KCN/SMS-KCNR, and CHLA-15/CHLA-20. Cell pellets were resuspended in 100 µl lysis buffer containing protease inhibitor cocktail (PIC, 1 µl/$1x10^6$ cells) and incubated on ice for 15 min, followed by centrifugation at 10,000x g for 30 min. Supernatant was transferred into a new Eppendorf tube, and protein concentration was determined using BioRad protein assay. Therefore, BioRad protein assay reagent was diluted 1:5 in aqua dest and added into a 96-well plate at a volume of 250 µl/well. 1 µl/well standard dilutions, ranging from 10 mg/ml to 0.15 mg/ml BSA, or 1 µl/well of sample was added to the respective wells. Samples were incubated for 5 min and analyzed with an ELISA reader at a wavelength of 595 nm.

After protein concentration was determined, 40 µg of protein were diluted 1:2 with Leammli sample buffer containing β-mercaptoethanol, and samples were denatured at 95°C for 5 min. SDS-PAGE was performed at 80 V for 20 min followed by 120 V for one hour, using a 10% resolving gel and a 4% stacking gel to resolve all proteins of the cell lysate. Fiber pads, filter paper and nitrocellulose membrane were incubated in transfer buffer for 20 min prior to blotting. The sandwich was assembled in the following order: fiber pad, filter paper, gel, membrane, filter paper, fiber pad. Afterwards, the blotting tank was filled with the sandwich, a cooling unit and transfer buffer, and blotting was then performed at 100 V for one hour. To block non-specific binding, the membrane was incubated with Rotiblock (1x solution in PBS) and shaken at room temperature for one hour. For detection of GCS, the membrane was incubated overnight while rotating at 4°C with mouse anti-human GCS antibody (1:200 diluted in Rotiblock). On the following day, the membrane was washed three times (10 min each) with washing buffer and was incubated with a HRP-labelled goat anti-mouse IgG antibody (1:2,000 in PBS) for one hour at room temperature. After washing the membrane three times (10 min each) with washing buffer, a 1:2 mixture of Immun-Star-HRP peroxide buffer and Immun-Star luminol/enhancer was added to the membrane, and the reaction was visualized using the ChemiDoc XRS™ Molecular Imager. The size of the detected GCS protein band (52 kDa) was determined according to a protein standard (Spectra™ Multicolor Broad Range Protein Ladder) on the membrane.

To compare protein expression between different samples, β-actin-expression was analyzed as a control. Therefore, the membrane was incubated with a HRP-labelled anti-β-actin antibody (1:30,000 in PBS) for one hour at room temperature. After washing the membrane three times (5 min each) with washing buffer, protein visualization was performed as described above. The ratio of GCS-expression in relapse cell lines to GCS expression in cell lines from diagnosis was determined according to the following formula: (intensity of GCS in relapse / intensity of GCS in diagnosis cell line) x (intensity of β-actin in diagnosis cell line / intensity of β-actin in relapse cell line).

2.2.7. *In vivo* efficacy of NK-92-scFv(ch14.18)-zeta in a drug-resistant NB mouse model

The *in vivo* efficacy of NK-92-scFv(ch14.18)-zeta was analyzed in a drug-resistant NB xenograft mouse model. Experiments were performed in compliance with the German Law for Welfare of Laboratory Animals. Since CHLA-20, NK-92-scFv(ch14.18)-zeta and NK-92-scFv(FRP5)-zeta are all human cell lines, an immunodeficient mouse model was chosen to ensure engraftment of subcutaneous tumors and prevent human NK cells from being attacked by mouse immune cells. Therefore female NSG (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ) mice were obtained at an age of 6-8 weeks from Charles River laboratories (Sulzfeld, Germany) and maintained under specific-pathogen free conditions. Mutation of the Prkdc gene affects recombination of VDJ segments in immunoglobulin genes and T cell receptor genes. Additionally, these mice exhibit a mutation in the gene encoding for the IL-2 receptor gamma chain. This IL-2 receptor gamma chain is important for high affinity binding and signaling of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Both mutations result in a mouse phenotype lacking mature B cells and T cells. This phenotype also displays decreased NK cell numbers and only very low cytotoxic activity of the remaining NK cells.

To determine the efficacy of NK-92-scFv(ch14.18)-zeta, mice were challenged with subcutaneous tumors of the drug-resistant human NB cell line CHLA-20 and subjected to eight peritumoral subcutaneous injections of a combination of NK cells and human recombinant IL-2. CHLA-20 tumor cells were harvested, washed twice in IMDM without supplements (300x g, 5 min, RT) and resuspended in a 1:2 mixture of PBS and Matrigel (BD Biosciences, Bedford, MA, USA). Matrigel is a basement membrane extract, generated from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. Extracellular matrix proteins and growth factors in matrigel promote engraftment and growth of xenografts [155]. Primary tumor growth of NB tumors was induced by subcutaneous injection of $1x10^6$ CHLA-20 into the left flank (day 0) of 28 mice. On day three after tumor cell inoculation, mice received the first peritumoral subcutaneous injection of $2x10^7$ NK cells and 200 IU IL-2 in 150 µl PBS. NK cells were washed twice in PBS prior to injection. One experimental

group (n=7) received a combination of NK-92-scFv(ch14.18)-zeta and IL-2. Control groups (n=7) were subjected to injection of PBS ("untreated") or NK-92-scFv(FRP5)-zeta + IL-2. Further, an additional control group (n=7) was injected with 200 IU IL-2 only. Since the parental NK-92 cell line, and therefore also the genetically engineered NK cell lines NK-92-scFv(ch14.18)-zeta and NK-92-scFv(FRP5)-zeta are IL-2 dependent, the application of IL-2 was included into the treatment schedule to ensure survival and stable expression of the CAR *in vivo.*

Mice received eight injections of the combination of NK cells and IL-2 in total, starting on day three after tumor cell inoculation (injections on day 3, 7, 11, 15, 19, 26, 33, 40). Additional intraperitoneal injections of IL-2 (1000 IU/mouse) were applied the day after each NK cell injection (days 4, 8, 12, 16, 20, 27, 34, 41) to both NK cell-treated groups as well as the IL-2 control group. Untreated mice received 100 µl PBS intraperitoneally on the respective days. To determine the effect of NK cell applications on subcutaneous primary tumor growth, tumor size was measured every other day using a micro caliper and tumor volume was calculated according to the following formula: length x $(width)^2$ x 0.5. Mice were sacrificed at a maximum tumor volume of 800 mm^3 or when tumors showed signs of necrosis.

2.2.8. Statistical analysis

Statistical analyses were done with GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). An unpaired t-test was used for comparison of two experimental groups, while statistic analysis of more than two experimental groups was performed using a one way ANOVA-test. Experimental differences were considered statistically significant if the resulting p-value was lower than 0.05 (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Statistical analysis of *in vivo* survival data was completed using a Log-rank (Mantel-Cox) test. To account for multiple comparison of survival curves, only p-values less than the Bonferroni-corrected significance level of 0.008 were considered significant (*$p<0.008$, **$p<0.002$, ***$p<0.0002$).

3. Results

3.1. GD2-specificity of NK-92-scFv(ch14.18)-zeta

3.1.1. Chimeric antigen receptor expression on NK-92-scFv(ch14.18)-zeta

To confirm GD2-specificity of NK-92-scFv(ch14.18)-zeta, CAR-expression was analyzed using flow cytometry. In this regard, two different staining strategies can be utilized for flow cytometric analysis of GD2-specific CAR-expression. The first is based on the use of an anti-idiotype antibody (anti-IdAb) called ganglidiomab, which has recently been generated and characterized as a surrogate of GD2. Due to its anti-idiotype characteristics, ganglidiomab is able to specifically bind the antigen binding regions of the GD2-specific CAR (Fig. 3.1.1 A). Therefore, both GD2-specificity and CAR-expression can be evaluated. The second staining strategy is based on an antibody recognizing a Myc-tag which is located in the extracellular part of the CAR (Fig. 3.1.1 B). While a positive staining of the Myc-tag indicates CAR-expression on NK cells, it does not provide any information about CAR-specificity. In addition to NK-92-scFv(ch14.18)-zeta, the control NK cell lines NK-92, NK-92-pLXSN as well as the ErbB2-specific NK-92-scFv(FRP5)-zeta were analyzed to confirm that they do not express a GD2-specific CAR (Fig. 3.1.1 C).

Staining of NK-92-scFv(ch14.18)-zeta using the anti-IdAb revealed that all NK-92-scFv(ch14.18)-zeta cells homogeneously express a CAR that specifically recognizes GD2. Myc-tag staining also showed CAR-expression on NK-92-scFv(ch14.18)-zeta. For parental NK-92 cells as well as the empty vector-transduced NK-92-pLXSN cell line, neither staining with anti-IdAb nor Myc-tag-specific antibody showed CAR-expression on the cell surface. Analysis of NK-92-scFv(FRP5)-zeta revealed a high and homogeneous CAR-expression after staining with the Myc-tag-specific antibody. Staining with anti-IdAb did not show any positive signal. This shows that the control cell line NK-92-scFv(FRP5)-zeta expresses a CAR that is not GD2-specific.

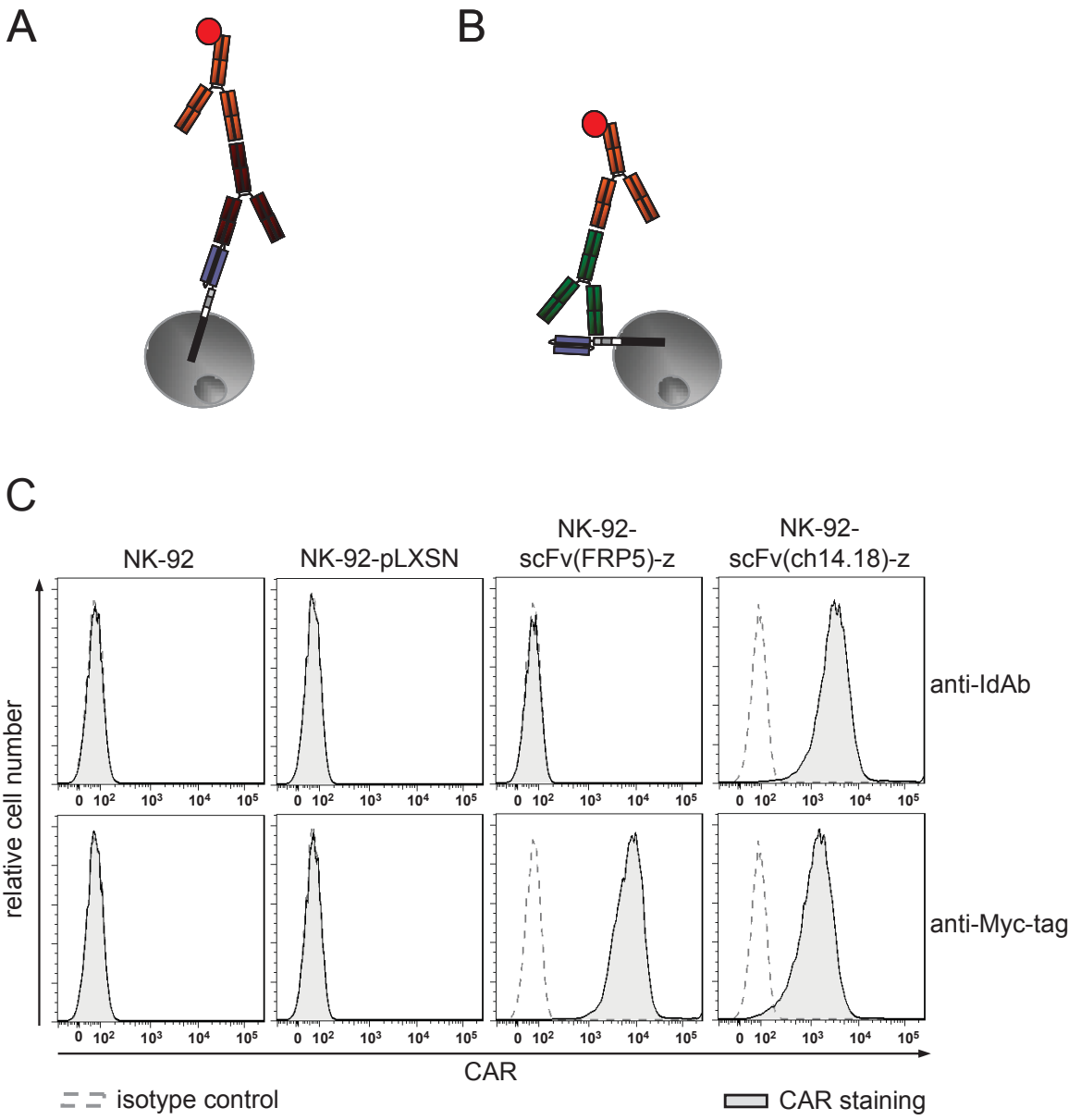


Figure 3.1.1: Chimeric antigen receptor expression on NK cell lines. Chimeric antigen receptor expression was analyzed with flow cytometry, based on two staining strategies. **(A)** CAR-detection by staining with 1 µg/1x10^6 cells anti-idiotype antibody ganglidiomab (red) and PE-labeled rat anti-mouse secondary antibody (orange). **(B)** CAR-detection based on recognition of the extracellular Myc-tag. NK cells were stained with 1 µg/1x10^6 cells anti-Myc-tag antibody (green) and PE-labeled rat anti-mouse secondary antibody (orange). **(C)** Representative histograms of CAR-staining with both staining strategies (isotype controls: gray dashed curves; CAR staining: filled black curve).

3.1.2. Cytotoxicity of NK-scFv(ch14.18)-zeta and control NK cell lines towards GD2$^+$ and GD2$^-$ NB cell lines

Calcein release cytotoxicity assays were performed to analyze GD2-specificity and functionality of the CAR expressed on NK-92-scFv(ch14.18)-zeta. To determine a suitable ratio of effector cells to target cells (E:T ratio) for these experiments, NK-92-scFv(ch14.18)-

zeta and the control cell line NK-92-scFv(FRP5)-zeta were applied at different E:T ratios (ranging from 0.8:1 to 50:1) in a calcein release cytotoxicity assay with the $GD2^+$ NB cell line CHLA-20 as target cells (Fig. 3.1.2.1). This experiment showed that NK-92-scFv(ch14.18)-zeta effectively lysed CHLA-20, compared to the control cell line NK-92-scFv(FRP5)-zeta and further that this effect was dependent on the E:T ratio. For the following cytotoxicity assays an E:T ratio of 6.3:1 was chosen, since the GD2-specific effect was clearly visible at this ratio and the amount of NK cells needed at this E:T ratio was applicable for complex experimental settings.

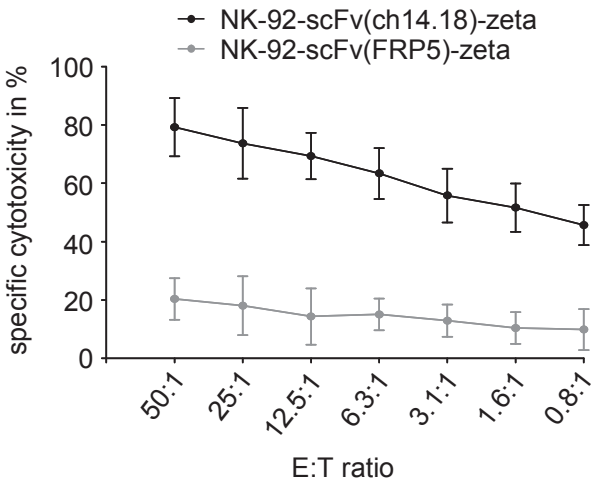


Figure 3.1.2.1: Determination of suitable E:T ratio for cytotoxicity assays. Specific cytotoxicities of NK-92-scFv(ch14.18)-zeta (black curve) and the control cell line NK-92-scFv(FRP5)-zeta (gray curve) were analyzed in a calcein release assay with the $GD2^+$ NB cell line CHLA-20 as target cells. To determine a suitable E:T ratio for further cytotoxicity assays, different E:T ratios in the range of 0.8:1 to 50:1 were tested. Results are presented as mean specific cytotoxicity ± SD from three independent experiments.

After a suitable E:T ratio was determined, NK-92-scFv(ch14.18)-zeta as well as all control NK cell lines were analyzed for their specific cytotoxicity towards the $GD2^+$ NB cell line CHLA-20 and the $GD2^-$ NB cell line SK-N-SH in calcein release assays. GD2-expression of target cell lines was confirmed with flow cytometry (Fig. 3.1.2.2 A). For comparison of independent experiments, calcein release data were normalized using NK-92-scFv(ch14.18)-zeta. Calcein assays revealed that specific cytotoxicities of NK-92-scFv(ch14.18)-zeta and control NK cell lines towards the GD2-negative NB cell line SK-N-SH at an E:T ratio of 6.3:1 were not statistically different from each other, with specific cytotoxicities ranging from 19% (NK-92-pLXSN) to 22% (NK-92) (Fig. 3.1.2.2 B). In contrast, NK-92-scFv(ch14.18)-zeta effectively lysed the GD2-expressing cell line CHLA-20 (specific cytotoxicity 55%), compared to control NK cell lines with cytotoxicities ranging from 22% for NK-92 to 25% for NK-92-scFv(FRP5)-zeta. These results clearly show that expression of the GD2-specific CAR on NK-92-scFv(ch14.18)-zeta results in a significantly higher cytotoxicity towards GD2-expressing cells compared to basal cytotoxicity of control NK cell lines. Further, comparison of all control NK cell lines revealed that their cytotoxicity is comparable. Therefore, only one NK control cell line was used as control in all following cytotoxicity assays.

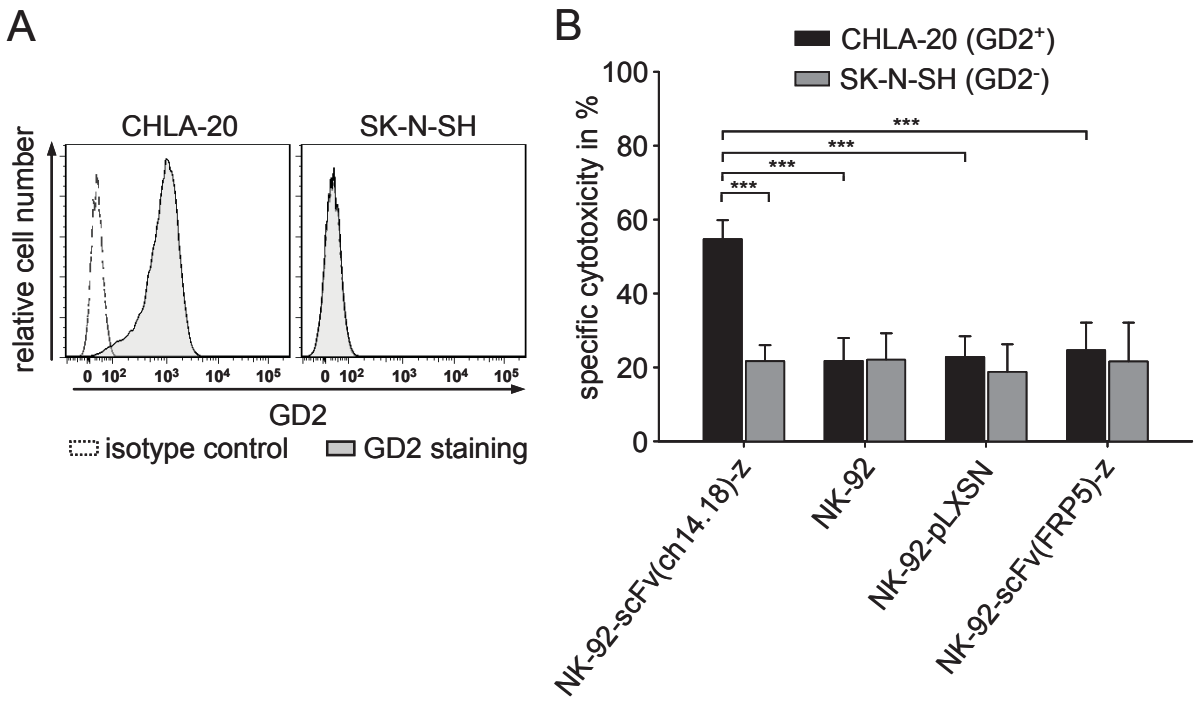


Figure 3.1.2.2: GD2-specificity of NK-92-scFv(ch14.18)-zeta. (A) GD2-expression on target cell lines CHLA-20 and SK-N-SH was analyzed with flow cytometry. Results are presented as one representative histogram for each cell line. Cells were stained with either 1 µg anti-GD2 antibody ch14.18 (filled black curve) or 1 µg isotype control (dashed black curve), followed by incubation with PE-labeled secondary antibody. **(B)** Cytotoxicity of NK-92-scFv(ch14.18)-zeta as well as control NK cell lines towards CHLA-20 and SK-N-SH was determined in calcein release assays at an E:T ratio of 6.3:1. NK-92-scFv(ch14.18)-zeta-mediated lysis of CHLA-20 was statistically different from lysis mediated by control NK cell lines (***p<0.001). Further, the difference in specific cytotoxicity of NK-92-scFv(ch14.18)-zeta towards CHLA-20 and SK-N-SH was statistically significant (***p<0.001).

3.2. Cytotoxicity of NK-92-scFv(ch14.18)-zeta towards GD2-expressing NB cell lines

Functionality and cytotoxic activity of NK-92-scFv(ch14.18)-zeta was further confirmed in ^{51}Cr release cytotoxicity assays with a panel of six different GD2-positive NB cell lines (CHLA-136, CHLA-79, CHLA-20, SK-N-BE(2), LA-N-1, LA-N-5), some of which exhibit partial or multidrug resistance. GD2-expression of all NB cell lines was confirmed by flow cytometry and results are shown in figure 3.2 A. To demonstrate GD2-specific lysis mediated by NK-92-scFv(ch14.18)-zeta, lysis mediated by control NK cells was set to zero as baseline level, and NK-92-scFv(ch14.18)-zeta-mediated lysis was normalized against lysis by control NK cell lines. Therefore, specific cytotoxicities presented in Figure 3.2 B represent the difference in specific lysis between the control NK cell line and NK-92-scFv(ch14.18)-zeta expressing the GD2-specific CAR.

^{51}Cr release assays revealed that NK-92-scFv(ch14.18)-zeta, at an E:T ratio of 6.3:1, effectively lysed all tested GD2-expressing NB cell lines, with specific cytotoxicities ranging from 34% (CHLA-79) to 66% (CHLA-136) (Fig. 3.2 B). For LA-N-5, CHLA-136, CHLA-79 and SK-N-BE(2), the empty vector control cell line NK-92-pLXSN was used as control, while parental NK-92 cells were utilized as control for the ^{51}Cr release assays with CHLA-20 and LA-N-1. These results show that GD2-specific CAR expression enables NK-92-scFv(ch14.18)-zeta to effectively lyse GD2-expressing NB cell lines.

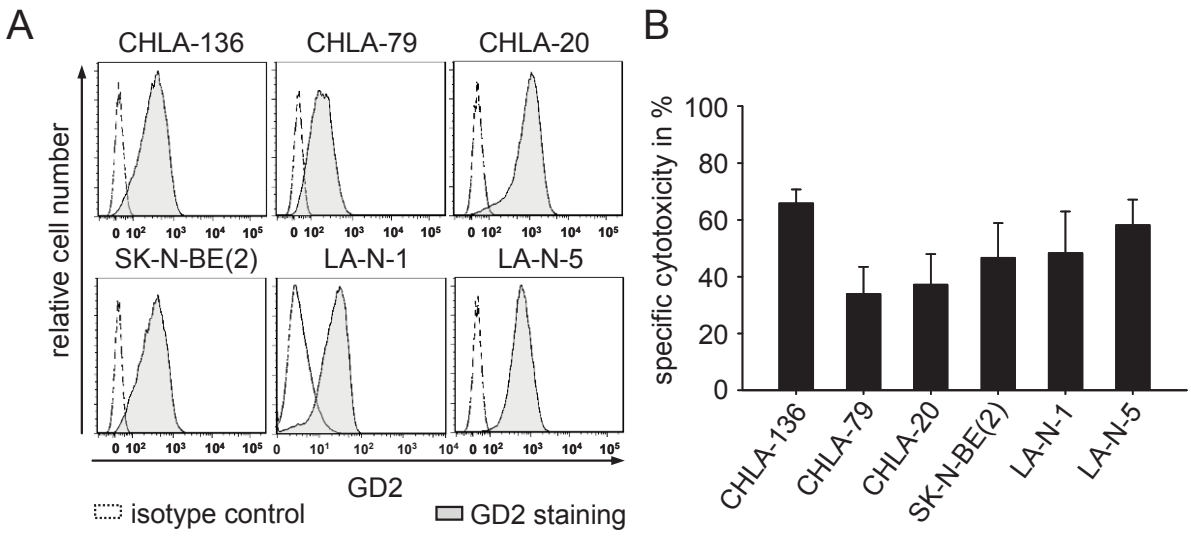


Figure 3.2: NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2-positive NB cell lines. (A) GD2-expression on six NB cell lines was analyzed using flow cytometry. Cells were stained with either 1 µg anti-GD2 antibody ch14.18 (filled curve) or 1 µg isotype control (dashed curve), followed by incubation with PE-labeled secondary antibody. Results are shown as one representative histogram for each cell line. **(B)** Cytotoxicity of NK-92-scFv(ch14.18)-zeta towards six GD2-positive NB cell lines at an E:T ratio of 6.3:1 was analyzed using ^{51}Cr-release assays. Specific cytotoxicity of NK-92-scFv(ch14.18)-zeta-mediated lysis was normalized against lysis by control NK cell lines. Results are presented as mean specific cytotoxicity ± SD from at least three independent experiments.

3.3. Role of GD2 recognition by chimeric antigen receptor for activation of NK-92-scFv(ch14.18)-zeta

To determine whether NK-92-scFv(ch14.18)-zeta-mediated cytotoxicity is dependent on recognition of the target antigen GD2, the CAR was blocked using the anti-IdAb ganglidiomab that specifically binds and blocks the antigen binding regions of the CAR. ^{51}Cr release assays were performed using the GD2^{+} NB cell lines LA-N-1 (Fig. 3.3.1 A) and LA-N-5 (Fig. 3.3.1 B) as target cells. Data from independent experiments were normalized against specific cytotoxicity of control samples without addition of any antibody to compare the effect of CAR-blocking on NK-92-scFv(ch14.18)-zeta-mediated lysis. In the case of LA-

N-1, addition of anti-IdAb significantly decreased specific cytotoxicity from 36% without addition of antibody to 8% with 10 µg/ml anti-IdAb. In contrast, addition of 10 µg/ml mouse IgG1 as isotype control did not result in decreased specific cytotoxicity. These observations were confirmed with LA-N-5 as target cells, resulting in a significant decrease in specific cytotoxicity from 49% in controls to 13% after addition of 10 µg/ml anti-IdAb. Addition of the same amounts of mouse IgG1 did not have a significant effect on specific cytotoxicity.

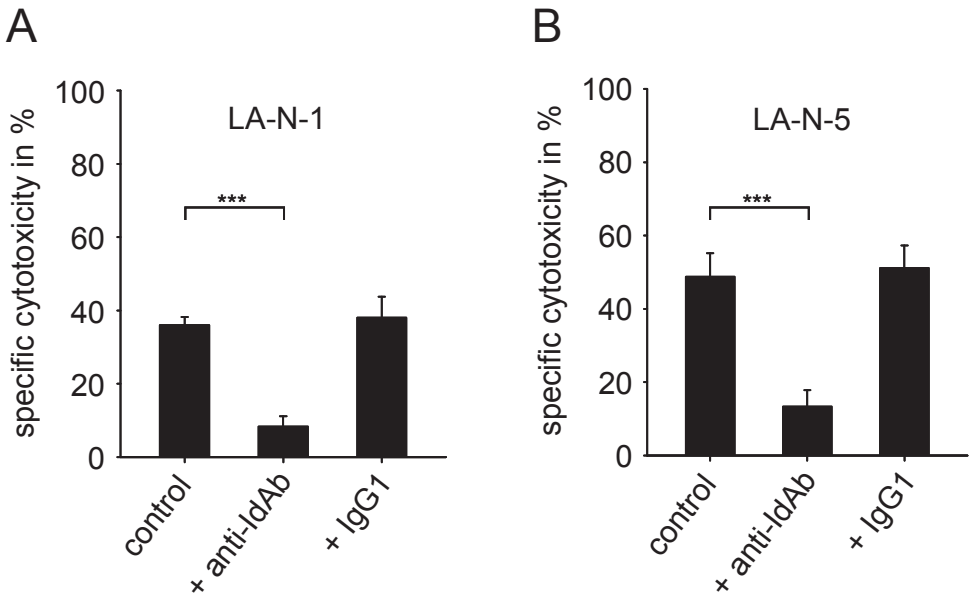


Figure 3.3.1: Blocking of the CAR expressed on NK-92-scFv(ch14.18)-zeta. GD2-specific CAR and thereby NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2-positive NB cell lines LA-N-1 **(A)** and LA-N-5 **(B)** was blocked in a ^{51}Cr-release assay by addition of 10 µg/ml anti-IdAb. For controls, respective amounts of mouse IgG1 were applied. Results are presented as mean specific cytotoxicity in % ± SD from at least three independent experiments. Data of independent experiments were normalized using specific cytotoxicity of control samples. Abrogation of NK-92-scFv(ch14.18)-zeta-mediated lysis of LA-N-1 and LA-N-5 by addition of anti-IdAb was statistically significant (***p< 0.001).

These results were further confirmed by CAR-blocking in calcein release cytotoxicity assays using the GD2$^+$ NB cell lines CHLA-15 and CHLA-20 as well as the GD2$^-$ erythroleukemia cell line K562 as target cells (Fig. 3.3.2). For comparison of independent experiments, calcein release data were normalized using control samples without addition of antibodies. These assays revealed that NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2-expressing CHLA-15 was almost completely abrogated by addition of 10 µg/ml anti-IdAb (38% specific cytotoxicity in controls compared to 6% with anti-IdAb), while addition of the same amounts of isotype control did not have any effect (Fig. 3.3.3 A). CAR-blocking by addition of anti-IdAb also induced almost complete abrogation of NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2$^+$ CHLA-20 (11% specific cytotoxicity with anti-IdAb compared to 40% specific cytotoxicity in controls). Addition of the same amounts of isotype control did

not have any effect (Fig. 3.3.3 B). In contrast, lysis of GD2-negative K562 was unaffected by addition of CAR-blocking anti-IdAb (37% specific cytotoxicity in controls compared to 36% with anti-IdAb and 37% with isotype control) (Fig. 3.3.3 C), indicating that this lysis is mediated by GD2-independent mechanisms.

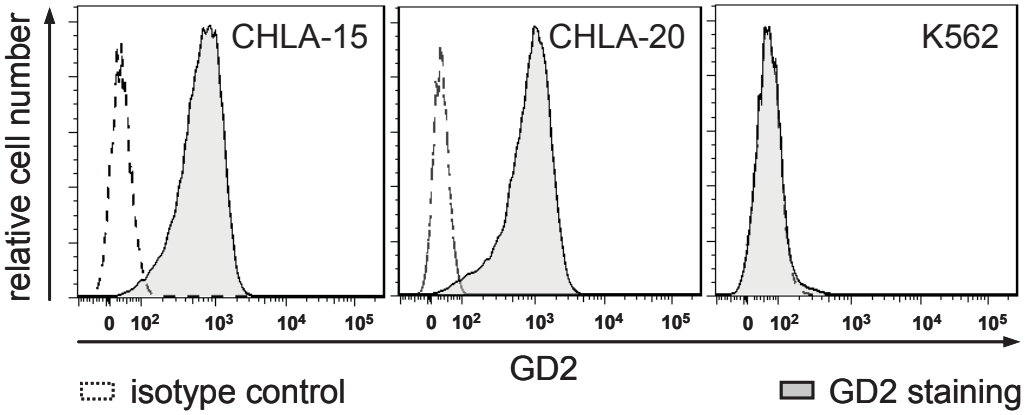


Figure 3.3.2: GD2 expression on NB cell lines CHLA-15 and CHLA-20 and erythroleukemia cell line K562. For flow cytometric analysis of GD2-expression, **c**ells were stained with either 1 µg anti-GD2 antibody ch14.18 (filled curve) or 1 µg isotype control (dashed curve), followed by incubation with PE-labeled secondary antibody. Results are shown as one representative histogram for each cell line.

These experiments demonstrate that NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2-expressing cell lines is based on recognition of GD2 by the GD2-specific CAR, expressed on the NK cell line. The almost complete abrogation of specific cytotoxicity of NK-92-scFv(ch14.18)-zeta towards $GD2^+$ cell lines after blocking the CAR further indicates that GD2-induced activation is the primary mechanism of target cell recognition and thereby NK-92-scFv(ch14.18)-zeta-mediated lysis.

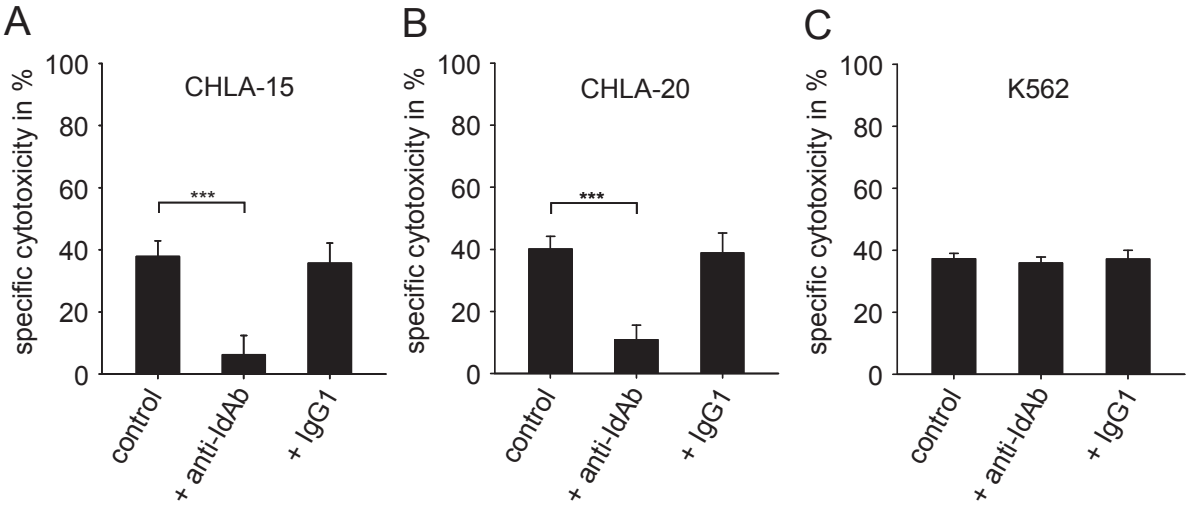


Figure 3.3.3: Effect of CAR-blocking on NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2$^+$ and GD2$^-$ target cell lines. CAR was blocked using 10 µg/ml anti-IdAb in a calcein release assay. Results are presented as mean specific cytotoxicity in % ± SD of at least three independent experiments. Data of independent experiments were normalized using specific cytotoxicity of control samples. **(A)** NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2$^+$ NB cell line CHLA-15 was abrogated by addition of 10 µg/ml anti-IdAb. This effect was statistically significant (***p<0.001). **(B)** Abrogation of NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2$^+$ NB cell line CHLA-20 by addition of 10 µg/ml anti-IdAb was statistically significant (***p<0.001). **(C)** CAR-blocking did not have any effect on NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2$^-$ cell line K562.

An alternative approach of disrupting the interaction of the target antigen GD2 on tumor cells and the CAR expressed on NK-92-scFv(ch14.18)-zeta is blocking the target antigen GD2 by addition of the GD2-specific antibody ch14.18. Importantly, NK-92 cells lack expression of FcγRs, and therefore addition of ch14.18 does not induce ADCC towards target cells. Ch14.18 was added during co-incubation of NK-92-scFv(ch14.18)-zeta and target cells in a calcein release cytotoxicity assay to further confirm that NK-92-scFv(ch14.18)-zeta-mediated lysis is dependent on recognition of GD2 (Fig. 3.3.4). CHLA-15 and CHLA-20 were used as GD2$^+$ NB cell lines target cells in these assays. Again, the erythroleukemia cell line K562 was utilized as negative control. Addition of 10 µg/ml ch14.18 during co-incubation resulted in abrogation of NK-92-scFv(ch14.18)-zeta-mediated lysis of CHLA-15 (13% specific cytotoxicity compared to 38% specific lysis in control without antibody) (Fig. 3.3.4 A). Addition of same amounts of rituximab as control did not have any effect. Comparable results were obtained with CHLA-20 as target cells (Fig. 3.3.4 B). Specific cytotoxicity of NK-92-scFv(ch14.18)-zeta was abrogated by addition of 10 µg/ml ch14.18 (14% specific cytotoxicity) compared to controls without addition of antibody (54% specific cytotoxicity) or addition of 10 µg/ml rituximab (57% specific cytotoxicity). In contrast, lysis of the negative control K562 was unaffected by addition of ch14.18 and thereby comparable in all samples (51% specific cytotoxicity in controls, 52%

and 54% specific lysis after addition of 10 µg/ml ch14.18 and same amounts of rituximab, respectively) (Fig. 3.3.4 C).

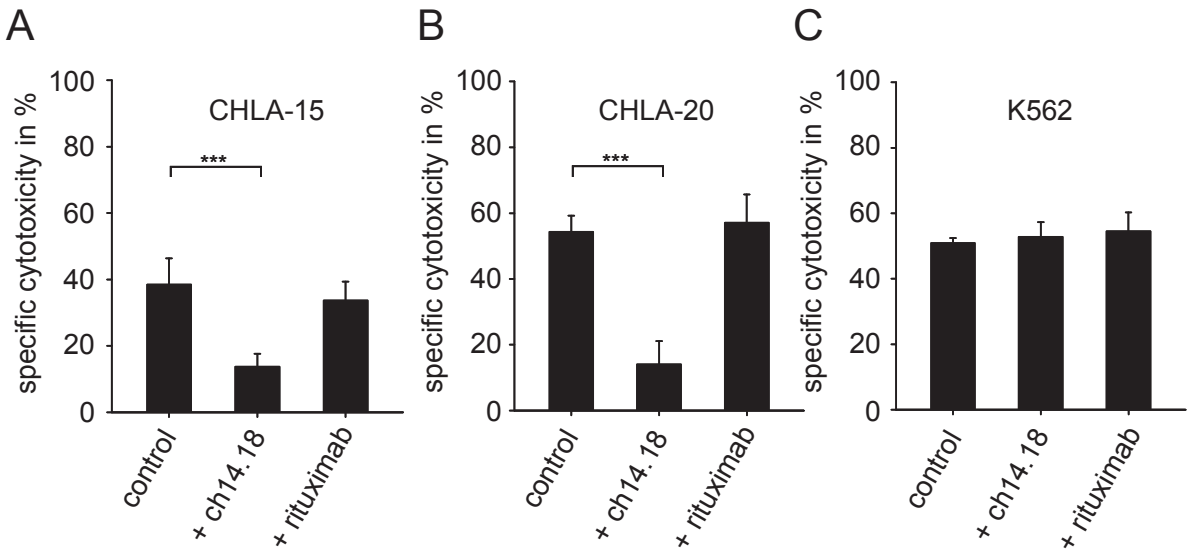


Figure 3.3.4: Effect of GD2-blocking on NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2$^+$ and GD2$^-$ target cell lines. GD2 on target cells was blocked using 10 µg/ml ch14.18 in a calcein release assay. Results are presented as mean specific cytotoxicity in % ± SD of three independent experiments. Data of independent experiments were normalized using specific cytotoxicity of control samples. **(A)** NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2$^+$ NB cell line CHLA-15 was abrogated by addition of 10 µg/ml ch14.18. This effect was statistically significant (***p<0.001). **(B)** Addition of 10 µg/ml ch14.18 resulted in abrogation of NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2$^+$ NB cell line CHLA-20. This effect was statistically significant (***p<0.001). **(C)** GD2-blocking did not have any effect on NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2$^-$ cell line K562.

Additionally, the impact of GD2 recognition by the CAR on activation of NK-92-scFv(ch14.18)-zeta was assessed using immobilized GD2. NK-92-scFv(ch14.18)-zeta were incubated with either immobilized target antigen GD2, or the ganglioside GM2 as a negative control. Activation of NK-92-scFv(ch14.18)-zeta was confirmed by measuring the concentration of effector molecules granzyme B (Fig. 3.3.5 A) and perforin (Fig. 3.3.5 B) in the supernatant, using ELISA. This experimental design excludes all possible stimuli that might be present on a target cell and potentially induce activation of NK-92-scFv(ch14.18)-zeta, such as activating NK cell ligands. Therefore, this assay provides a possibility to analyze the effect of GD2 on the activation of NK-92-scFv(ch14.18)-zeta. Incubation with immobilized GD2 resulted in a significant 6-fold increase in granzyme B and a 2.5-fold increase in perforin release, compared to NK-92-scFv(ch14.18)-zeta controls. In contrast, incubation with GM2 did not have a significant effect on granzyme B or perforin release of NK-92-scFv(ch14.18)-zeta. This confirms GD2-specificity of NK-92-scFv(ch14.18)-zeta and shows that GD2 as a stimulus is sufficient to induce activation of CAR-expressing NK-92-scFv(ch14.18)-zeta.

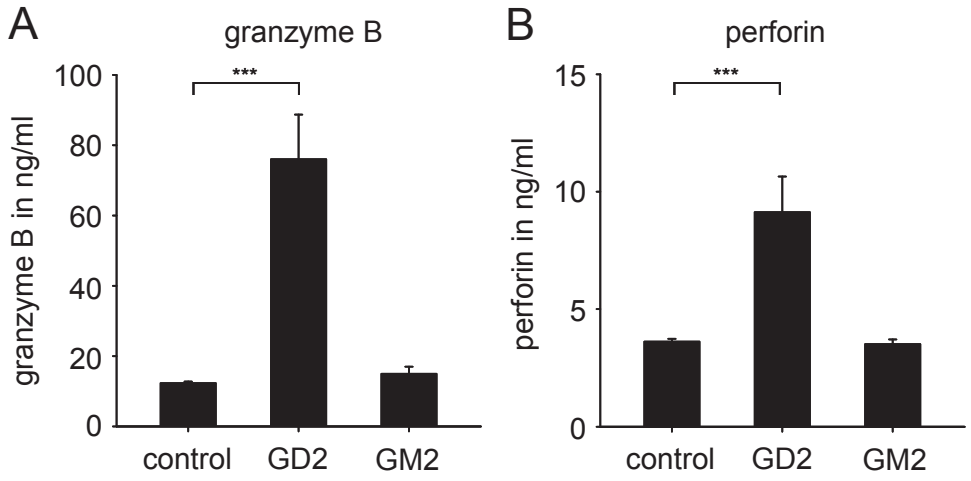


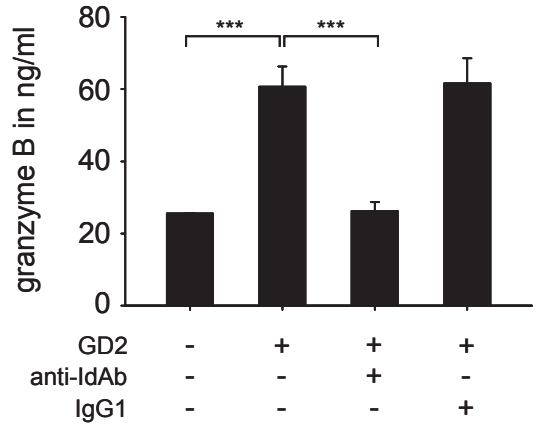
Figure 3.3.5: Release of effector molecules granzyme B and perforin in response to immobilized gangliosides. NK-92-scFv(ch14.18)-zeta were incubated with 100 ng GD2 or GM2 for five hours and effector molecules granzyme B **(A)** and perforin **(B)** were quantified in supernatants using ELISA. Results are presented as mean effector molecule concentration in ng/ml ± SD from three independent experiments. Data of independent experiments were normalized according to NK-92-scFv(ch14.18)-zeta that were incubated without immobilized gangliosides (control). Differences in granzyme B and perforin release in response to incubation with immobilized GD2 were statistically significant (***p<0.001).

To show that the GD2-induced increase in effector molecule release of NK-92-scFv(ch14.18)-zeta is dependent on GD2 recognition by the CAR, the receptor was blocked by addition of 10 µg/ml anti-IdAb in the presence of immobilized GD2. Concentrations of effector molecules in supernatants were then determined using ELISA. The 2.4-fold increase in granzyme B release in the presence of immobilized GD2, compared to NK-92-scFv(ch14.18)-zeta incubated in the absence of gangliosides (control), could be completely abrogated by addition of anti-IdAb (3.3.6 A and B). Addition of 10 µg/ml IgG1 isotype control in the presence of immobilized GD2 still resulted in a 2.4-fold increase. Furthermore, incubation of control NK cell line NK-92-pLXSN in the presence of GD2 had no effect on granzyme B release, while both NK cell lines reacted to non-specific activation with PMA/Ionomycin (5.3-fold increase in NK-92-scFv(ch14.18)-zeta and 4.6-fold increase in NK-92-pLXSN) (Fig. 3.3.6 B). Analysis of perforin release of NK-92-scFv(ch14.18)-zeta revealed similar results (Fig. 3.3.6 C and D). Incubation of NK-92-scFv(ch14.18)-zeta in the presence of immobilized GD2 resulted in a 1.6-fold increase in perforin release compared to NK-92-scFv(ch14.18)-zeta incubated in the absence of GD2. This GD2-induced increase could be abrogated by addition of anti-IdAb, in contrast to addition of isotype control (1.7-fold increase in perforin production). Again, NK-92-pLXSN did not respond to activation with GD2, while both NK cell lines reacted to non-specific

activation with PMA/Ionomycin (2.2-fold increase in NK-92-scFv(ch14.18)-zeta and 3.8-fold increase in NK-92-pLXSN) (Fig. 3.3.6 D).

These results show that the expression of the GD2-specific CAR enables NK-92-scFv(ch14.18)-zeta to specifically respond to activation with GD2 in contrast to the control NK cell line NK-92-pLXSN. Furthermore, results demonstrate that stimulation with the target antigen GD2 is sufficient to induce activation of NK-92-scFv(ch14.18)-zeta, thereby suggesting an antigen-specific stimulation as the primary mechanism of activation of NK-92-scFv(ch14.18)-zeta, in the absence of other possible activating stimuli that might be present on a target cell.

A

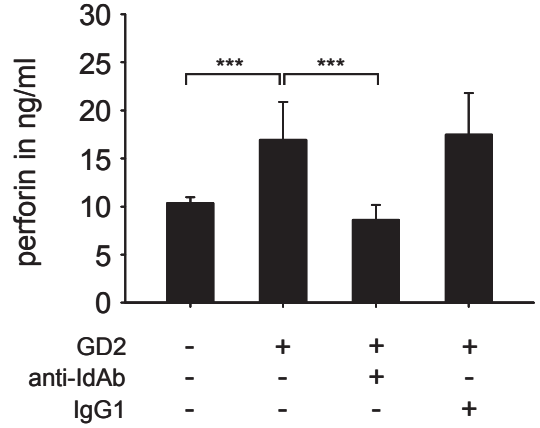


B

Fold increase in granzyme B compared to control

	NK-92tr	NK-92-pLXSN
PMA/I	5.3 ± 0.8	4.6 ± 0.4
GD2 only	2.4 ± 0.2	0.9 ± 0.1
+ anti-IdAb 10 µg/ml	1.0 ± 0.1	0.7 ± 0.1
+ IgG1 10 µg/ml	2.4 ± 0.3	0.8 ± 0.1

C



D

Fold increase in perforin compared to control

	NK-92tr	NK-92-pLXSN
PMA/I	2.2 ± 0.8	3.8 ± 0.5
GD2 only	1.6 ± 0.4	1.0 ± 0.2
+ anti-IdAb 10 µg/ml	0.8 ± 0.2	0.8 ± 0.1
+ IgG1 10 µg/ml	1.7 ± 0.4	0.9 ± 0.1

Figure 3.3.6: Effect of CAR-blocking on release of effector molecules granzyme B and perforin. NK-92-scFv(ch14.18)-zeta and the NK control cell line NK-92-pLXSN were incubated in the presence or absence of 100 ng GD2 for five hours and the concentration of effector molecules granzyme B and perforin was determined in supernatant using ELISA. To determine the effect of blocking the GD2-specific CAR, 10 µg/ml anti-IdAb or respective amounts of mouse IgG1 as isotype control were added during incubation. As a control, PMA/Ionomycin was used for non-specific activation of NK cells. To compare independent experiments, data were normalized according to basal release of effector molecules in the absence of GD2. **(A)** Mean granzyme B release of NK-92-scFv(ch14.18)-zeta in ng/ml ± SD of three independent experiments. Changes in granzyme B release in response to stimulation with GD2 as well as abrogation of granzyme B release by addition of anti-IdAb were statistically significant (***$p<0.001$). **(B)** Fold increase in granzyme B production of NK-92-scFv(ch14.18)-zeta and NK-92-pLXSN compared to respective controls incubated in the absence of GD2. Results are presented as mean fold increase ± SD of three independent experiments. **(C)** Mean perforin release of NK-92-scFv(ch14.18)-zeta in ng/ml ± SD of three independent experiments. Increase in perforin production in response to stimulation with GD2 as well as abrogation of perforin release by addition of anti-IdAb were statistically significant (***$p<0.001$). **(D)** Fold increase in perforin production of NK-92-scFv(ch14.18)-zeta and NK-92-pLXSN compared to respective controls incubated in the absence of GD2. Results are presented as mean fold increase ± SD of three independent experiments.

The impact of GD2 antigen recognition on NK-92-scFv(ch14.18)-zeta-mediated lysis was further directly analyzed by blocking ganglioside synthesis. Therefore, two GD2-expressing cell lines (SK-N-BE(2), CHLA-20) were treated with the selective glucosylceramide synthase (GCS) inhibitor PPPP (1-phenyl-2-hexadecanoylamino-3-pyrollidino-1-propanol). Glucosylceramide synthase is the first enzyme in ganglioside synthesis and mediates the conversion of ceramides to glucosylceramides. The effect of GCS-inhibition on GD2-expression was confirmed using flow cytometry. Figure 3.3.7 A shows representative histograms of GD2 expression on PPPP- as well as vehicle-treated SK-N-BE(2) and CHLA-20 and the respective mean fluorescence intensities (MFI). To compare GD2-expression levels between independent experiments, the mean fluorescence intensity ratios (MFI-ratio) were calculated according to the following formula: mean fluorescence intensity of GD2-stained sample / mean fluorescence intensity of isotype control. Results of five independent experiments are presented in Figure 3.3.7 B as the mean MFI ratio ± SD for PPPP- and vehicle-treated SK-N-BE(2) and CHLA-20, demonstrating that GCS-inhibition results in significantly reduced GD2 surface expression.

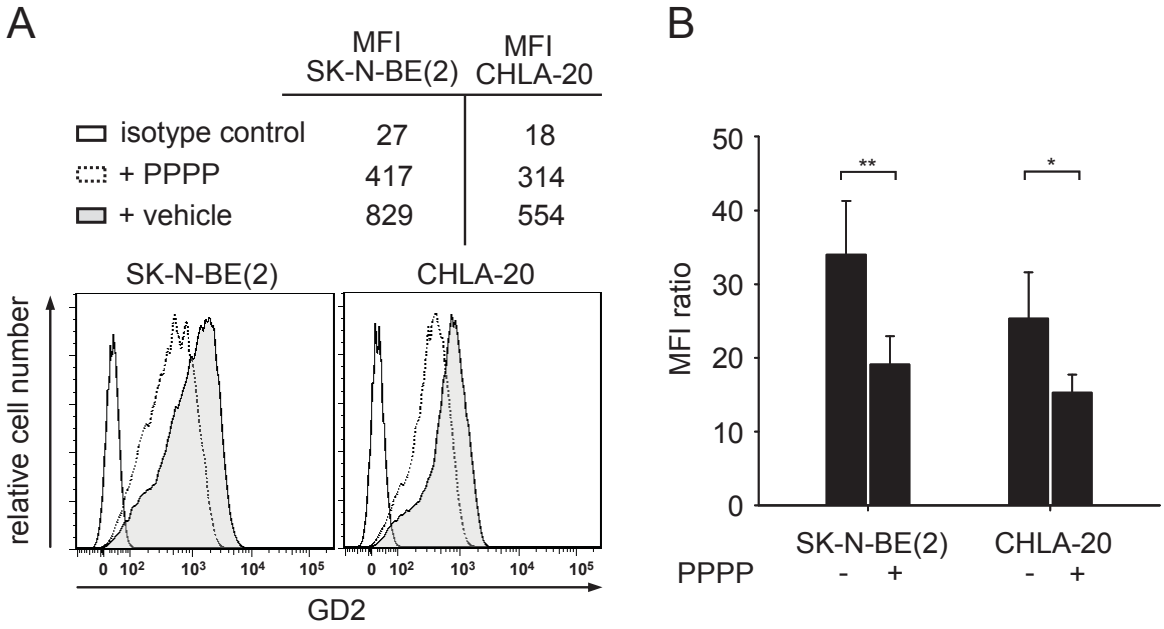


Figure 3.3.7: Effect of GCS-inhibition on GD2 surface expression of NB cell lines. SK-N-BE(2) and CHLA-20 were treated with 1 µM PPPP or the same volume of 100% ethanol (vehicle control) for 48 h and GD2 expression was analyzed with flow cytometry. **(A)** Representative histograms of GD2-expression on PPPP- (dashed black curve) and vehicle-treated (filled black curve) SK-N-BE(2) and CHLA-20. Controls were stained with rituximab as isotype control (black curve). **(B)** MFI ratios of GD2-expression on PPPP- and vehicle-treated SK-N-BE(2) and CHLA-20. MFI ratio was calculated according to the formula: mean fluorescence intensity of GD2-stained sample divided by mean fluorescence intensity of isotype control. PPPP-treatment resulted in significantly decreased GD2 surface expression on SK-N-BE(2) (**p=0.0037) and CHLA-20 (*p=0.01), compared to vehicle-treated cells. Results are presented as mean MFI ratio ± SD of five independent experiments.

Furthermore, application of PPPP- and vehicle-treated SK-N-BE(2) and CHLA-20 target cells in a ^{51}Cr release assay revealed that decreased GD2 surface expression correlated with decreased sensitivity of PPPP-treated SK-N-BE(2) and CHLA-20 towards NK-92-scFv(ch14.18)-zeta-mediated lysis (14% and 38% specific cytotoxicity, respectively), compared to vehicle-treated cells (39% and 63% specific cytotoxicity, respectively) (Fig. 3.3.8).

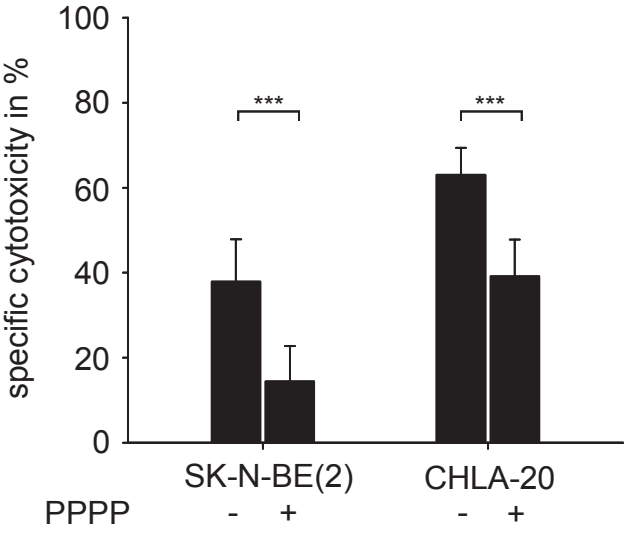


Figure 3.3.8: Effect of GCS-inhibition on NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2$^+$ NB cell lines. SK-N-BE(2) and CHLA-20 were treated with 1 µM PPPP or the same volume of 100% ethanol (vehicle control) for 48 h. Sensitivity towards NK-92-scFv(ch14.18)-zeta-mediated lysis was analyzed in a ^{51}Cr release assay. PPPP-treatment of SK-N-BE(2) and CHLA-20 resulted in significantly reduced sensitivity towards NK-92-scFv(ch14.18)-mediated cytotoxicity (***p<0.001). Results are presented as mean specific cytotoxicity in % ± SD of three independent experiments. To compare independent experiments, data were normalized to respective vehicle-treated control.

3.4. Sensitivity of NB relapse cell lines towards NK-92-scFv(ch14.18)-zeta-mediated lysis

Despite the use of intensive multi-modal therapy protocols, prognosis for high-risk NB patients remains poor. The treatment of high-risk NB patients is particularly challenging, because many patients initially respond to therapy but develop progressive disease or relapse, which is potentially caused by minimal residual disease. Unfortunately, relapsed tumors often exhibit drug resistance and are refractory towards chemotherapy. Therefore, alternative approaches for treatment of minimal residual disease are needed.

To address this problem, the sensitivity of NB cell line pairs from four patients towards NK-92-scFv(ch14.18)-zeta-mediated lysis was analyzed in ^{51}Cr release assays. Each cell line pair consists of one cell line established at time of diagnosis (SK-N-BE(1), SMS-KAN, SMS-KCN, CHLA-15) and a corresponding cell line generated from relapse material (SK-N-BE(2), SMS-KANR, SMS-KCNR, CHLA-20) of the same patient. For comparison of independent experiments, specific cytotoxicity of control NK cell lines was set to zero and specific cytotoxicity of NK-scFv(ch14.18)-zeta was normalized against control NK cell lines.

^{51}Cr release assays revealed that relapse cell lines of all patients tested are still sensitive towards NK-92-scFv(ch14.18)-zeta-mediated lysis, with specific cytotoxicities ranging from 32% (SMS-KANR) to 47% (SK-N-BE(2) (Fig. 3.4.1). Interestingly, in three out of four tested cell line pairs (SK-N-BE(1)/(2), SMS-KAN/SMS-KANR, SMS-KCN/SMS-KCNR), the relapse cell line showed significantly higher sensitivity towards NK-92-scFv(ch14.18)-zeta compared to the respective cell line from time point of diagnosis. Relapse cell line SK-N-BE(2) exhibited a 2-fold higher sensitivity compared to SK-N-BE(1). Sensitivity of SMS-KANR towards NK-92-scFv(ch14.18)-zeta was about 1.9-fold higher than sensitivity of SMS-KAN. In case of SMS-KCN/SMS-KCNR, the relapse cell line SMS-KCNR showed an almost 1.5 fold higher sensitivity. The fourth patient cell line pair (CHLA-15/CHLA-20) exhibited comparable sensitivities towards NK-92-scFv(ch14.18)-zeta.

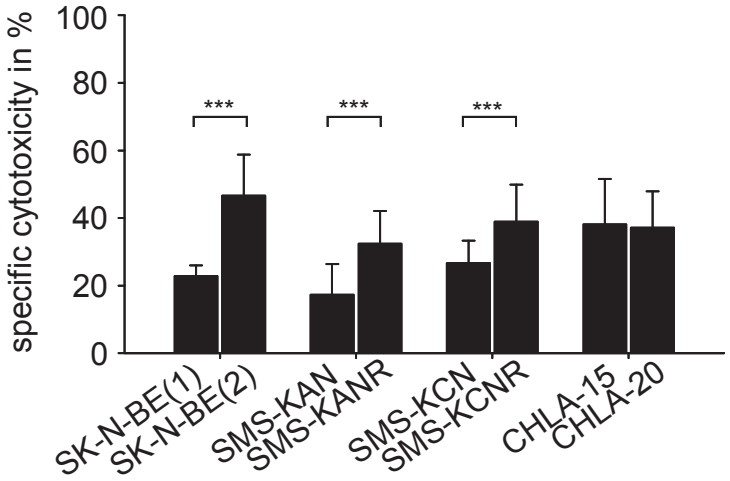


Figure 3.4.1: Sensitivity of NB cell line pairs towards NK-92-scFv(ch14.18)-zeta-mediated lysis. NB cell line pairs of four patients were analyzed in ^{51}Cr release assays. NK-92-scFv(ch14.18)-zeta effectively lysed all relapse cell lines at an E:T ratio of 6.3:1. In three out of four patients tested (SK-N-BE(1)/(2), SMS-KAN/SMS-KANR, SMS-KCN/SMS-KCNR), the relapse cell line exhibited a significantly higher sensitivity towards NK-92-scFv(ch14.18)-mediated lysis (***p≤0.001), compared to the respective cell line from time point of diagnosis. Specific cytotoxicity of control cell lines was set to zero and NK-92-scFv(ch14.18)-zeta-mediated lysis was normalized against control NK cells. Results are presented as mean specific cytotoxicity in % ± SD of at least three independent experiments.

Since recognition of GD2 by the CAR was shown to be the main mechanism of NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2-positive NB cell lines, cell line pairs from all four patients were analyzed for GD2 surface expression. Figure 3.4.2 A displays representative histograms of GD2-expression on NB cell line pairs. To compare GD2 expression levels of independent experiments, mean fluorescence intensity ratios (MFI-ratio) were calculated according to the following formula: MFI of GD2-stained sample divided by MFI of isotype-stained sample. Results of four independent experiments are

presented in Figure 3.4.2 B as the mean MFI ratio ± SD. Flow cytometric analysis of GD2 surface expression revealed that, in three out of four cases tested, the relapse cell line exhibited significantly higher GD2-expression compared to the respective cell line from time point of diagnosis. In the case of CHLA-15/CHLA-20, GD2-expression on the relapse cell line was not significantly higher. Thus, higher GD2-expression in relapse cell lines SK-N-BE(2), SMS-KANR and SMS-KCNR correlated with increased sensitivity of these cell lines towards NK-92-scFv(ch14.18)-zeta-mediated lysis in ^{51}Cr release assays.

These results show that relapse cell lines are sensitive towards NK-92-scFv(ch14.18)-zeta-mediated lysis, because they still express the antigen GD2. In our tests, the majority of relapse cell lines even displayed significantly increased GD2-expression, correlating with an increased specific cytotoxicity mediated by NK-92-scFv(ch14.18)-zeta.

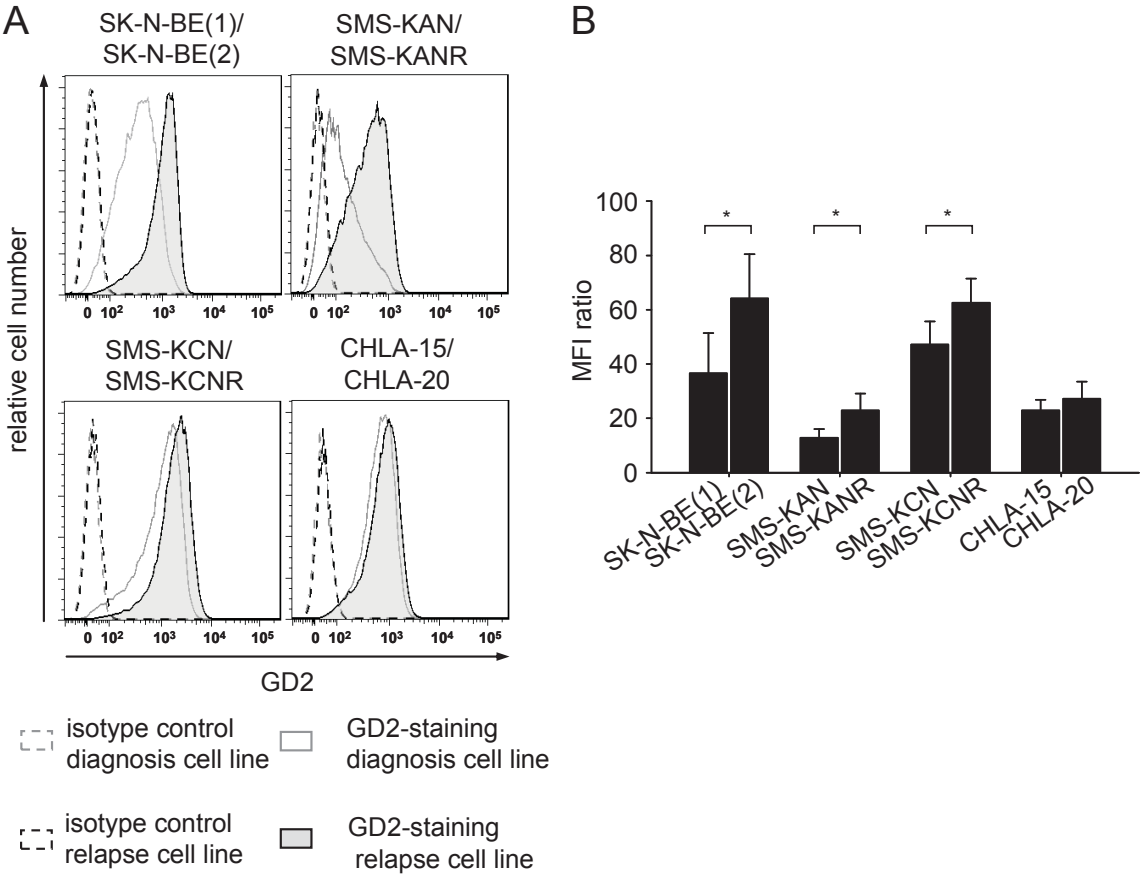
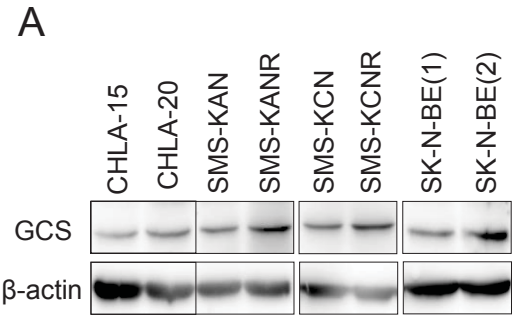


Figure 3.4.2: GD2-expression on NB cell line pairs. GD2-expression on NB cell line pairs of four patients was determined with flow cytometry. **(A)** Representative histograms for each cell line pair. (gray dashed curve: isotype control of diagnosis cell line; gray solid curve: GD2-staining of diagnosis cell line; black dashed curve: isotype control of relapse cell line; filled black curve: GD2-staining of relapse cell line). **(B)** MFI ratios of GD2-expression NB cell line pairs. MFI ratio was calculated according to the formula: mean fluorescence intensity of GD2-stained sample divided by mean fluorescence intensity of isotype control. Relapse cell lines SK-N-BE(2), SMS-KANR and SMS-KCNR exhibited a significantly higher GD2-expression than respective diagnosis cell lines (*$p<0.05$). Results are presented as mean MFI ratio ± SD of four independent experiments.

Increased expression of GCS is associated with relapse cell lines and a common mechanism of drug resistance that is activated in tumor cells to avoid accumulation of ceramides and evade apoptosis [156-158]. Since GCS as the first enzyme in GD2 synthesis was shown to have an effect on GD2 surface expression and thereby on NK-92-scFv(ch14.18)-zeta-mediated lysis (Fig. 3.3.6 and 3.3.7), GCS-expression in NB cell line pairs was analyzed using Western blot. All relapse cell lines showed a higher GCS-expression compared to their respective diagnosis cell lines (Fig. 3.4.3 A). Based on the β-actin control, GCS-expression was normalized for all cell lines and the ratio of GCS expression relapse/diagnosis cell line was calculated. To compare results from independent experiments for each cell line pair, mean ratios were calculated (Fig. 3.4.3 B). Results showed that the relapse cell lines SMS-KANR, SMS-KCNR and SK-N-BE(2) exhibited higher GCS-expression, while expression in the relapse cell line CHLA-20 was only slightly increased compared to diagnosis cell line CHLA-15. These results correlate with GD2-expression on NB cell line pairs, suggesting that increased GCS-expression might be one possible explanation for higher sensitivity of relapse cell lines SMS-KANR, SMS-KCNR and SK-N-BE(2) towards NK-92-scFv(ch14.18)-zeta.



B

Ratio of GCS expression relapse/diagnosis cell line

CHLA-20/CHLA-15	1.27 ± 0.49
SMS-KANR/SMS-KAN	1.54 ± 0.15
SMS-KCNR/SMS-KCN	1.88 ± 0.67
SK-N-BE(2)/SK-N-BE(1)	1.61 ± 0.47

Figure 3.4.3: GCS-expression in NB cell line pairs. GCS-expression in NB cell line pairs of four patients was analyzed using Western blot. For relative quantification of GCS-expression, β-actin-expression was analyzed as control. **(A)** Representative Western blots for GCS-expression (top row) as well as β-actin control (bottom row) in NB cell line pairs. **(B)** Ratio of GCS-expression of relapse to diagnosis cell lines. To compare GCS-expression in relapse and diagnosis cell lines, GCS-expression was normalized using the respective β-actin control, and intensity of relapse cell line was divided by intensity of diagnosis cell line. Results are presented as mean ratio of GCS-expression relapse to diagnosis cell lines ± SD from at least three independent experiments.

3.5. *In vivo* efficacy of NK-92-scFv(ch14.18)-zeta in a drug-resistant NB mouse model

Since NK-92-scFv(ch14.18)-zeta was effective towards a panel of $GD2^+$ NB cell lines *in vitro*, the in *vivo* efficacy of a therapeutic application of NK-92-scFv(ch14.18)-zeta was analyzed in a drug-resistant NB xenograft mouse model.

Prior to the actual *in vivo* study, the effect of IL-2 starvation on CAR-expression was examined in an *in vitro* experiment to determine whether an application of IL-2 should be included in the *in vivo* experiment. Therefore, NK-92-scFv(ch14.18)-zeta were cultured under different conditions, containing culture in complete medium, starvation from IL-2 for four days and culture in the absence of IL-2 for four days and subsequent transfer to complete medium for two additional days. CAR-expression was then analyzed in all samples with flow cytometry (Fig. 3.5.1). This analysis revealed that starvation from IL-2 resulted in decreased CAR-expression compared to controls cultured in complete medium. Abrogated CAR-expression was reversed by culture of cells in complete medium for two additional days. This suggests that an application of IL-2 should be included in the experimental setting of the following *in vivo* study.

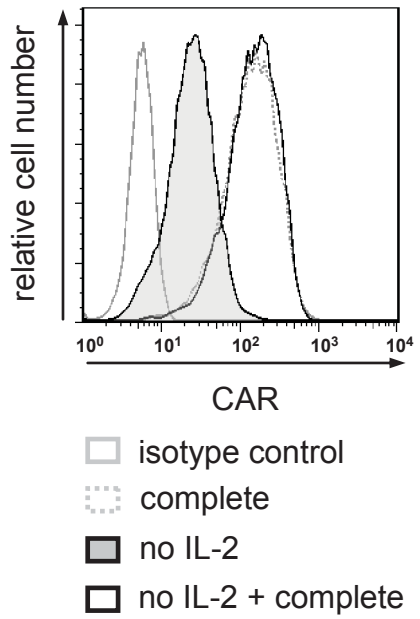


Figure 3.5.1: Impact of IL-2-starvation on CAR-expression on NK-92-scFv(ch14.18)-zeta. To assess the impact of IL-2 on CAR-expression, NK-92-scFv(ch14.18)-zeta were cultured under different culture conditions and CAR-expression was analyzed by staining with the anti-IdAb ganglidiomab. Mouse IgG1 was used as isotype control (gray curve) for staining. Cells were cultured in either complete medium (dashed gray curve), IL-2-free medium for four days (black filled curve), or IL-2-free medium for four days followed by two additional days in complete medium (black curve). Results are presented as a representative histogram.

Therapeutic efficacy of NK-92-scFv(ch14.18)-zeta was then determined according to their effect on the subcutaneous primary tumor growth of $GD2^+$ CHLA-20 tumors (Fig. 3.5.2). The primary tumor was induced by subcutaneous injection of $1x10^6$ CHLA-20 NB cells into the left flank of female NSG mice (day 0). Starting on day three after tumor inoculation, mice were subjected to a total of eight peritumoral injections of NK-92-scFv(ch14.18)-zeta and 200 IU of recombinant human IL-2 (injections on day 3, 7, 11, 15, 19, 26, 33, 40). Additionally, mice received 1000 IU of IL-2 intraperitoneally 24 h after each NK cell injection (injections on day 4, 8, 12, 16, 20, 27, 34, 41). To specifically show that the mediated effect of a repeated application of NK-92-scFv(ch14.18)-zeta is based on the expression of the GD2-specific CAR on these cells, the experimental setting also included control groups treated with PBS only (untreated) or either IL-2 only or a combination of the control NK cell line NK-92-scFv(FRP5)-zeta and IL-2. Since NK-92-scFv(ch14.18)-zeta and

NK-92-scFv(FRP5)-zeta are IL-2-dependent cell lines and IL-2-dependency of CAR-expression had been demonstrated in a previous *in vitro* experiment, the application of IL-2 was included in the treatment schedule to ensure survival and stable CAR-expression of NK cell lines.

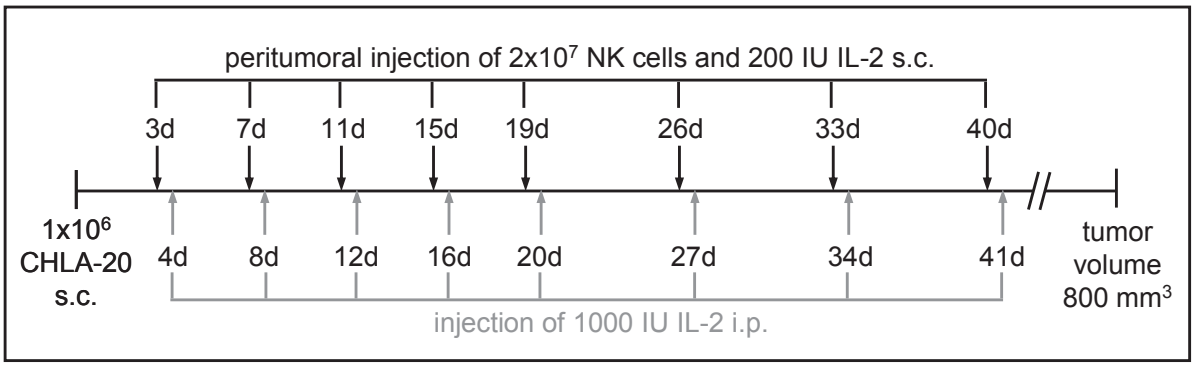


Figure 3.5.2: Treatment schedule for therapeutic treatment with NK-92-scFv(ch14.18)-zeta. $1x10^6$ CHLA-20 were subcutaneously injected into the left flank of female NSG (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ) mice. Mice received eight peritumoral subcutaneous (s.c.) injections of $2x10^7$ NK cells and 200 IU human recombinant IL-2 in total, starting on day three after tumor cell inoculation (days 3, 7, 11, 15, 19, 26, 33, 40). Additionally, mice were subjected to intraperitoneal (i.p.) injections of 1000 IU IL-2 24 h after each NK cell injection (days 4, 8, 12, 16, 20, 27, 34, 41). Experimental groups (n=7) included mice treated with PBS only (untreated), IL-2 only, NK-92-scFv(ch14.18)-zeta + IL-2 as well as a control group treated with NK-92-scFv(FRP5)-zeta + IL-2.

Peritumoral injections of NK-92-scFv(ch14.18)-zeta resulted in delayed appearance of palpable tumors in treated mice (mean time to appearance 30 days) compared to control groups (mean time to appearance 13-20 days). Although growth of primary CHLA-20 tumors was quite aggressive, treatment with GD2-specific NK-92-scFv(ch14.18)-zeta effectively delayed tumor growth. To demonstrate this effect, experimental groups were compared based on time until the tumors reached a volume of ≥150 mm^3 (Fig. 3.5.3). This comparison revealed that tumor growth was significantly delayed in NK-92-scFv(ch14.18)-zeta-treated mice (49 days) compared to control groups (27-29 days).

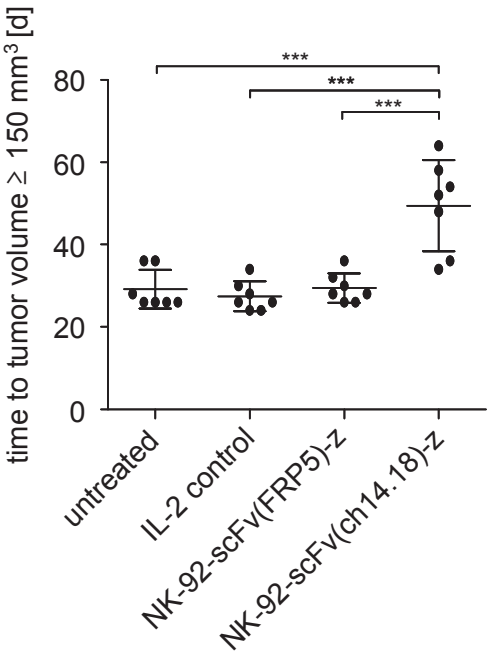


Figure 3.5.3: Comparison of tumor growth based on time to tumor volume ≥ 150 mm^3. Tumor growth was followed up by micro-caliper measurements and tumor volume was calculated according to the formula: length x (width)^2 x 0.5. Results are presented as mean time in days until an established tumor with a volume of ≥ 150 mm^3 was developed ± SD (n=7). Time until the tumors reached a volume ≥ 150 mm^3 was statistically longer in NK-92-scFv(ch14.18)-zeta-treated mice (***p<0.001).

Since tumor growth in control groups was comparable, Figure 3.5.4 shows tumor growth of individual mice in the NK-92-scFv(FRP5)-zeta control and NK-92-scFv(ch14.18)-zeta-treated groups as well as the treatment schedule. Interestingly, the overlay of tumor growth of individual mice of both groups showed that mice in the NK-92-scFv(ch14.18)-zeta group were sacrificed at a smaller tumor size than NK-92-scFv(FRP5)-zeta-treated mice. To confirm this finding, the mean tumor volumes on the day mice were sacrificed of all groups were compared. This comparison revealed that NK-92-scFv(ch14.18)-zeta-treated mice were sacrificed at a lower tumor volume (348 ± 124 mm^3) than control mice (untreated group 605 ± 101 mm^3, IL-2 group 635 ± 128 mm^3, NK-92-scFv(FRP5)-zeta group 666 ± 86 mm^3). Mice of the NK-92-scFv(ch14.18)-zeta-treated group had to be sacrificed before they reached the maximum tumor size of 800 mm^3 due to development of necrosis in these tumors at a lower tumor volume compared to tumors in control groups. This might be a sign for increased tumor tissue destruction potentially caused by an increased invasion and killing of tumor cells by NK-92-scFv(ch14.18)-zeta.

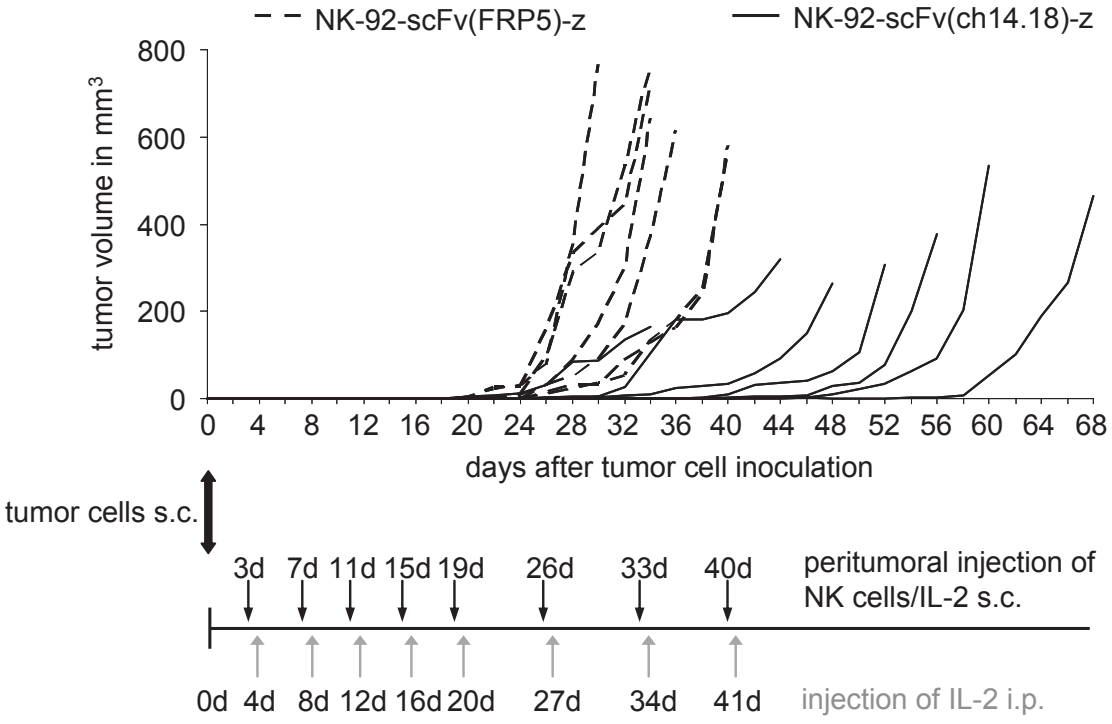


Figure 3.5.4: Tumor growth of individual mice of NK-92-scFv(ch14.18)-zeta-treated group and NK-92-scFv(FRP5)-zeta control group. Mice were challenged with subcutaneous injection of $1x10^6$ CHLA-20 in the left flank (day 0). Starting on day three, mice received peritumoral subcutaneous injections of either a combination of $2x10^7$ NK-92-scFv(ch14.18)-zeta and 200 IU IL-2 or $2x10^7$ NK-92-scFv(FRP5)-zeta and 200 IU IL-2 (days 3, 7, 11, 15, 19, 26, 33, 40). Additional intraperitoneal injections of 1000 IU IL-2 were performed 24 h after each NK cell injection (days 4, 8, 12, 16, 20, 27, 34, 41). Tumor volume was calculated according to the formula: length x $(width)^2$ x 0.5. Results are presented as the tumor volume of individual mice (n=7) of the NK-92-scFv(ch14.18)-zeta-treated group (black solid curves) and the NK-92-scFv(FRP5)-zeta control group (black dashed curves).

Most importantly, survival analysis of all groups showed, that the repeated therapeutic application of NK-92-scFv(ch14.18)-zeta resulted in significantly higher median survival (52 days) compared to controls (untreated mice 30 days, IL-2 control 30 days, NK-92-scFv(FRP5)-zeta group 32 days) (Fig. 3.5.5).

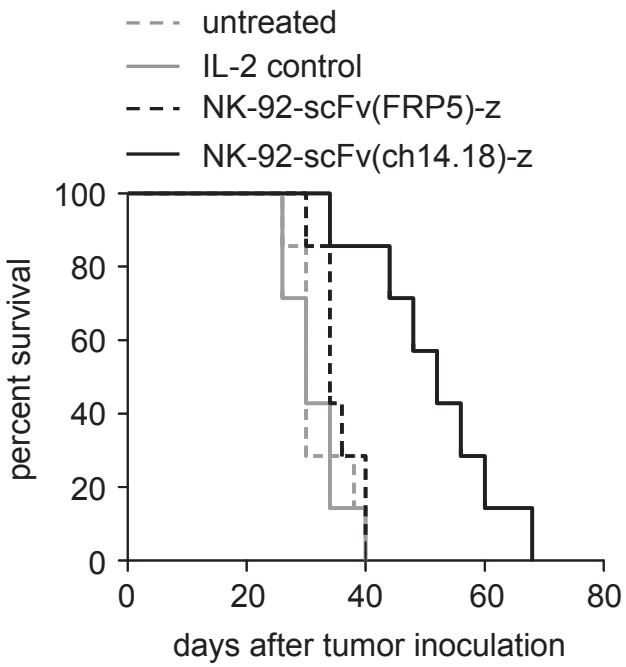


Figure 3.5.5: Therapeutic effect of repeated peritumoral NK cell applications on survival. Primary tumors were induced by subcutaneous injection of $1x10^6$ CHLA-20 into the left flank of mice (day 0). Starting on day three, mice received a total of eight injections of $2x10^7$ NK-92-scFv(ch14.18)-zeta + 200 IU IL-2 (days 3, 7, 11, 15, 19, 26, 33, 40). Additionally, mice were subjected to intraperitoneal injections of 1000 IU IL-2 24 h after each NK cell injection (days 4, 8, 12, 16, 20, 27, 34, 41). Controls were injected with PBS only (untreated), IL-2 only or NK-92-scFv(FRP5)-z and IL-2. Results are presented as percent survival (n=7) of untreated mice (gray dashed curve), IL-2-treated mice (gray solid curve), mice of the NK-92-scFv(ch14.18)-zeta group (black solid curve) and mice of the NK-92-scFv(FRP5)-zeta group (black dashed curve). Survival of NK-92-scFv(ch14.18)-zeta-treated mice was significantly higher compared to untreated mice (**p=0.0009), IL-2-treated mice (**p=0.0008) and NK-92-scFv(FRP5)-zeta-treated mice (*p=0.002). There was no statistically significant difference in survival of control groups.

4. Discussion

4.1. Immunotherapy in NB

Neuroblastoma (NB) is an aggressive childhood malignancy, with about 50% of patients exhibiting a high-risk phenotype associated with poor prognosis [2]. Despite intensive therapy protocols based on radiation, chemotherapy, stem cell support and differentiation therapy, standard treatment fails to successfully control high-risk NB in the majority of patients, resulting in the development of progressive disease or relapse. The induction of drug resistance in response to intensive therapy is a major obstacle to treatment with conventional therapies. Hence, the development and refinement of effective alternative therapies are of great importance. In this respect, immunotherapy is increasingly attractive. Clinical evidence of the potential of immunotherapy in NB was demonstrated in a phase III trial conducted by the Children's Oncology Group in the US, based on a protocol including a combination of the GD2-specific antibody ch14.18, IL-2, GM-CSF and 13-cis-retinoic acid [74]. This new treatment approach resulted in a significant increase in the two year event-free survival rate from 46 to 66%. Although this shows the potential of immunotherapy in NB, it also emphasizes the need for further improvement of therapeutic regimens, since about 30% of high-risk patients still do not survive the disease.

The most advanced immunotherapeutic approaches in NB are based on targeting the disialoganglioside GD2. Due to its abundant expression on NB cells but restricted physiological expression in the brain and peripheral nerve fibers [29], GD2 is a suitable antigen for passive immunotherapy with specific antibodies. With regard to the development of evasion mechanisms by the tumor, it is important that GD2 was shown not to be modulated off the cell membrane in response to GD2-targeted immunotherapy [31]. A combination of antibody-based immunotherapy and adoptive transfer of immune effector cells might further increase the therapeutic efficacy of immunotherapeutic approaches targeting GD2.

4.2. Cellular therapy and chimeric antigen receptors

According to the missing self hypothesis, low or absent MHC class I expression renders NB cells sensitive to NK cell lysis. Therefore, the use of NK cells as immune effector cells for adoptive transfer is attractive. In general, cellular therapy with NK cells can be based on reinfusion of *ex vivo* stimulated and expanded autologous NK or lymphokine

activated killer cells (LAK cells), or the application of allogeneic cells in particular in a haploidentical setting.

Clinical responses to a therapeutic application of autologous NK or LAK cells have been only moderate [159-161]. The match of MHC class I molecules on tumor cells and inhibitory KIR expressed on NK cells is a factor that can negatively affect the efficacy of therapy with autologous NK cells. Importantly, due to independent inheritance of KIR and HLA class I genes, HLA/KIR mismatch can also happen in the autologous setting. This means that a person expresses a certain KIR but simultaneously lacks expression of the respective HLA class I molecule, recognized by that KIR. This autologous KIR/HLA mismatch has been shown to be beneficial in terms of improved outcome of patients [162]. A retrospective study in high-risk NB patients who received an autologous HSCT demonstrated that KIR/HLA mismatch is a prognostic factor for an improved outcome of patients [163]. Another factor that has to be considered for the application of autologous cells is the observation that the functionality of NK cells can be impaired in cancer patients. Different mechanisms have been suggested to be involved in this phenomenon [summarized in 164]. One is a decreased expression of activating NK cell receptors, such as NCRs or NKG2D [95, 165]. Furthermore, decreased expression or impaired function of the signal-transducing zeta chain in patient T cells and NK cells has been reported for various malignancies, such as cervical cancer, colorectal carcinoma, ovarian carcinoma, prostate cancer and myeloid malignancies [166-170]. Additionally, redistribution of NK cell subsets in favor of the less cytotoxic $CD16^{dim}$ $CD56^{bright}$ has been reported for cancer patients [171, 172].

With regard to these problems, the use of allogeneic cells (particularly haploidentical NK cells) is appealing. The mismatch between MHC class I molecules on the patients' tumor cells and inhibitory KIRs on donor NK cells in an allogeneic setting is beneficial to activation of NK cells due to the lack of inhibitory signaling [173, 174]. Additionally, NK cells expanded from a healthy donor might exhibit improved functionality compared to the patient's NK cells. Clinical applications of allogeneic haploidentical NK cells in particular in the setting of hematopoietic stem cell transplantation, have shown more promising results [175-180].

Independent of the autologous or allogeneic nature of NK cells applied within an immunotherapeutic approach, tumor cells can develop escape strategies to evade recognition by NK cells. This includes shedding as well as down regulation of activating ligands or release of soluble factors (such as TGF-β), eventually resulting in down regulation of activating receptors on NK cells or their respective ligands on tumor cells [37-42, 181]. The application of a chimeric antigen receptor (CAR) in order to specifically direct

NK cells to tumor target cells and, at the same time, provide for an activation signal following specific targeting of a TAA is an approach to overcome these evasion strategies. In the case of NB, the disialoganglioside GD2 is an established target antigen for passive immunotherapy based on the application of GD2-specific antibodies. Chimeric antigen receptors have been generated to combine antibody-based immunotherapeutic approaches and the adoptive transfer of immune effector cells to increase the therapeutic potential. In general, CARs are not dependent on presentation of antigens in the context of MHC class I molecules and therefore are able to recognize carbohydrate or glycolipid antigens in addition to protein antigens. Importantly, the expression of chimeric antigen receptors provides effector cells with a certain specificity and thereby overcomes the above mentioned immune evasion strategies, since lysis is not only dependent on interaction of activating NK cell receptors with their respective ligands on tumor cells anymore.

In comparison to the application of anti-GD2 antibodies, the use of CAR-expressing effector cells might be especially beneficial for patients who only express Fc receptors for IgG (FcγR) with low affinity for the therapeutic antibody. This correlation between the affinity of the FcγR and clinical efficacy of antibody-based immunotherapy has been reported across a variety of therapeutic antibody applications [182-185]. Interestingly, studies indicated that the use of CAR-expressing effector cells might compare favorably to the use of a combination of monoclonal antibodies and NK cells [148]. Patients who did not respond to therapy with the chimeric bispecific antibody blinatumomab (anti-CD19 and anti-CD3) were shown to respond to CAR-expressing effector cells [186]. Furthermore, immunotherapeutic studies based on the application of the GD2-specific immunocytokine hu14.18-IL2 have shown correlations between HLA/KIR mismatch and improved outcome [187, 188]. In addition to the potential benefit of a CAR-based approach compared to the application of an TAA-specific antibody, this also suggests that the application of a CAR-expressing allogeneic cell line might be especially beneficial for patients who do not exhibit an autologous HLA/KIR mismatch status.

Another challenge to passive immunotherapy with monoclonal antibodies is that patients are very often heavily pre-treated with high-dose multi-agent chemotherapy. This application of cytostatic agents negatively affects numbers and cytotoxic activity of endogenous immune cells, resulting in immune suppression of patients. In this respect, the adoptive transfer of CAR-engineered allogeneic effector cells is becoming increasingly attractive as a substitute for functionally impaired endogenous immune cells, thereby suggesting a greater therapeutic potential for immunotherapeutic applications.

Cellular therapy based on the adoptive transfer of CAR-expressing effector cells, mainly T cells, has already entered clinical trials. So far, approaches using 1st generation

CARs have shown only modest success [189-191]. Interestingly, the exception was a phase I clinical trial in NB patients treated with autologous T cells that were genetically engineered to express a GD2-specific CAR [192]. In detail, 11 patients received a combination of CAR-expressing activated T cells (ATC) and CAR-expressing CTLs that are specific for Epstein Barr-virus (EBV), intended to provide additional co-stimulation. CAR-expressing CTLs were detectable at six weeks after injection, while CAR expressing ATC were only detectable until three weeks after injection. Four out of eight patients that entered the study with detectable disease responded with tumor necrosis or tumor regression after treatment with GD2-specific autologous T cells [192]. Long-term monitoring of these patients and eight additional patients demonstrated comparable persistence of both effector cell populations at a low level for up to four years [146]. Persistence of either cell type correlated with prolonged time to disease progression in 11 patients with active disease. Three out of these 11 patients experienced complete response. Importantly, in addition to these responses, this phase one trial demonstrated that the therapeutic application of CAR-expressing effector cells was safe and well tolerated. According to clinical trials based on the therapeutic application of GD2-specific antibodies, neuropathic pain is a major side effect due to physiological expression of GD2 on peripheral nerve fibers. This side effect was not reported upon treatment with GD2-CAR-expressing effector cells. So far, studies showed that neuropathic pain is potentially caused by recognition of GD2 on peripheral nerve fibers, and subsequent activation of the complement system, based on the interaction between complement proteins and the Fc part of the anti-GD2 antibody [193]. This suggests that in the case of GD2-targeting therapeutic strategies, the use of CAR-expressing effector cells might be beneficial compared to the application of GD2-specific antibodies, because the CAR does not contain a Fc part and therefore does not activate the complement system. Furthermore, it was reported that treatment of patients with GD2-CAR-expressing effector cells did not induce the generation of antibodies against the CAR [146].

To address the problem of low persistence and expansion of 1st generation CAR-expressing effector cells which resulted in only modest therapeutic effects, effector cells expressing a 2nd generation CAR including a co-stimulatory domain have been developed and entered clinical trials [reviewed in 194-196]. So far, the most promising results regarding persistence and expansion of CAR-expressing effector cells and clinical response have been demonstrated towards CD19^{+} hematological malignancies [145, 186, 197, 198]. Kochenderfer *et al.* [197] reported results of a clinical application of CD28-CD3ζ CAR-T cells specifically targeting CD19. Out of eight patients, five experienced partial remission and one patient showed complete remission. In the majority of patients CAR-T

cells were detectable up to 50 days, while in some patients CAR-T cells persisted for 130 days or longer. Persistence of CD19 specific 4-1BB-CD3ζ CAR-T cells for up to six months was reported by Kalos *et al.* [145] in three patients with chronic lymphocytic leukemia (CLL). Importantly, CAR-T cells expanded more than 1000-fold after infusion into patients. Two of these patients experienced complete remission, while a third patient showed a partial response. Porter *et al.* [198] reported results of the treatment of one additional CLL patient with 4-1BB-CD3ζ CAR-T cells. This patient also experienced complete remission of disease and showed greater than 1000-fold expansion of CAR-T cells which persisted for six months. These promising results were confirmed by the use of 4-1BB-CD3ζ CAR-T cells in two patients with acute lymphoid leukemia (ALL) [186]. Expansion of CAR-T cells greater than 1000-fold as well as persistence of cells for six months and longer was confirmed in both patients. Treatment with 4-1BB-CD3ζ CAR-T cells resulted in complete remission in both patients, with one patient maintaining complete remission after nine months. The other patient relapsed two months after treatment.

Not many 3rd generation CARs, which include several co-stimulatory domains have entered clinical trials so far. A CD20-specific 3rd generation CAR containing CD28 and 4-1BB co-stimulatory domains was utilized in a pilot clinical trial [147]. Out of four patients with follicular lymphoma or mantle cell lymphoma that enrolled to the study, three patients received CAR-expressing T cells. Two of these patients entered the study without evaluable disease and remained progression-free for 12 and 24 months after treatment. The third patient entered partial remission but relapsed 12 months after treatment. Importantly, CAR-expression on infused modified T cells was only detectable with PCR and persistence of cells in patients was confirmed up 12 months after treatment. In contrast, the application of T cells expressing a 3rd generation ErbB2-specific CAR, containing CD28 and 4-1BB co-stimulatory domains, in a patient with colorectal cancer caused a serious adverse event resulting in death [199].

All of the above mentioned CAR-based immunotherapeutic approaches have utilized T cells as effector cells. Since the understanding of NK cell biology and regulation of NK cell activation is evolving, the use of NK cells as alternative effector cells is becoming increasingly attractive. In contrast to T cells, NK cells mediate a non-MHC-restricted natural cytotoxicity without any prior sensitization [80, 83]. Importantly, this natural cytotoxicity of NK cells has already been shown to play a role in the lysis of NB cells [35, 36, 200]. Although T cells are advantageous with regard to initial number of effector cells which can be isolated from peripheral blood, progress has been made in the establishment of protocols for NK isolation and *ex vivo* expansion and activation, compensating for the initial lower cell count. These improved protocols are based on the expansion of NK cells using

an artificial antigen presenting cell line generated by genetic engineering of K562 to express one or several activating ligands, such as CD137L or MICA and IL-15 or IL-21 as a membrane-bound activating cytokine [201-204]. Within several expansion cycles, NK cell numbers can be expanded up to 47,967-fold depending on the artificial antigen presenting cell line that is used. These new and improved NK cell expansion and activation protocols potentially compensate the initial difference in cell numbers of T cells and NK cells. Importantly, NK cells might be advantageous as effector cells compared to T cells in an allogeneic adoptive transfer setting. It has been shown that NK cells mediate cytotoxicity in the graft versus tumor direction but do not cause graft-versus-host disease (GvHD), a serious complication that is potentially mediated by T cells [177, 205, 206]. The shorter life span of NK cells compared to T cells might be beneficial with regard to long-term side effects which can be potentially mediated by memory-like T cells [207]. Such a long-term side effect has been reported in clinical trials based on the application of CD19-specific CAR-expressing T cells, which resulted in long-term depletion of normal B cells [197, 198].

Based on these advantages of NK cells, the approach of CAR-based immunotherapy has already been expanded to NK cells as effector cells [133, 143, 208]. Although these studies demonstrated the feasibility of a CAR-based approach employing NK cells as effector cells, they also demonstrated that there are still limitations regarding donor-dependent extensive variations in transfection efficacy and CAR functionality [133, 143]. Furthermore, *ex vivo* expansion, activation and genetic engineering of autologous or allogeneic primary NK cells for each individual patient is time intensive and requires substantial resources.

The human NK cell line NK-92 is a promising alternative to address the problems associated with the use of primary NK cells. In contrast to primary patient-derived NK cells that might be functionally impaired, NK-92 provides unrestricted alloreactivity due to lack of expression of KIRs [119]. NK-92 has been shown to exhibit a high cytotoxicity against a variety of cancer cell lines and primary cancer cells [116-118]. As a prerequisite for a clinical application large scale expansion of NK-92 can be easily performed under good manufacturing practices (GMP) [121, 122]. NK-92 expressing CARs of various specificities have already been generated [140, 142, 148, 209, 210], demonstrating the feasibility of genetic engineering of NK-92. Once CAR-expressing NK-92 of certain specificity are generated, they can be provided on demand in a standardized quality and can be used not only for one specific patient but many different patients. This non-individualized approach is advantageous compared to the use of patient-derived effector cells, because it compensates for functional impairment of primary effector cells as well as donor-dependent variations in *ex vivo* expansion rates, transfection efficacy and CAR-

functionality. Most importantly, the characteristic of being ready on demand in a standardized quality circumvents the obstacle of time and labor consuming isolation, transfection, expansion and testing of patient-derived effector cells. This reduction in lead time is an important prerequisite for the initiation of multicenter clinical trials in the maintenance therapy immediately after conventional therapy to prevent a relapse.

To expand this approach to NB, this thesis aimed to analyze the mechanism and efficacy of a cellular therapy based on a human NK cell line expressing a GD2-specific CAR (NK-92-scFv(ch14.18)-zeta).

4.3. Effect and mechanism of a GD2-directed NK CAR therapy approach based on NK-92-scFv(ch14.18)-zeta

The initial part of this study involved the analysis of CAR-expression on NK-92-scFv(ch14.18)-zeta as well as the control cell lines NK-92, NK-92-pLXSN, and NK-92-scFv(FRP5)-zeta with flow cytometry, because CAR-expression is the prerequisite for GD2-specific lysis of target cells. High homogenous CAR-expression was confirmed for NK-92-scFv(FRP5)-zeta and NK-92-scFv(ch14.18)-zeta, with only NK-92-scFv(ch14.18)-zeta expressing a GD2-specific CAR (Fig. 3.1.1). *In vitro* cytotoxicity assays demonstrated significantly higher lysis of GD2-expressing NB cell lines mediated by NK-92-scFv(ch14.18)-zeta compared to control NK cell lines (Fig. 3.1.2-3.2). As the development of drug-resistance remains a major challenge in NB treatment with conventional therapies, the panel of GD2-positive target cell lines tested in these assays also included NB cell lines, which are known to be partially or even multidrug-resistant. This was intended to assess the sensitivity of NB cell lines towards a GD2-targeted approach with NK-92-scFv(ch14.18)-zeta. NK-92-scFv(ch14.18)-zeta effectively lysed all tested NB cell lines, even those with a drug-resistant phenotype, indicating that targeting GD2 with a cellular approach based on NK-92-scFv(ch14.18)-zeta might offer an alternative therapeutic approach to address the problem of acquired drug-resistance with conventional therapies.

The role of the interaction between the target antigen GD2 and the CAR for cytotoxic activity of NK-92-scFv(ch14.18)-zeta was assessed in additional *in vitro* cytotoxicity assays by disrupting the interaction, either by blocking the CAR with an anti-IdAb (Fig. 3.3.1 and 3.3.2) or by blocking the target antigen GD2 by addition of the GD2-specific antibody ch14.18 (Fig. 3.3.3) during co-incubation of NK-scFv(ch14.18)-zeta and target cells. Both strategies resulted in significant abrogation of NK-92-scFv(ch14.18)-zeta-mediated lysis of GD2-positive target cells. Importantly, lysis was almost completely abrogated, indicating that recognition of GD2 by the CAR is the primary mechanism of activation of NK-92-scFv(ch14.18)-zeta-mediated lysis. To directly analyze the impact of GD2-expression on

NK-92-scFv(ch14.18)-zeta-mediated lysis of NB cell lines, GD2-expression on NB target cells was decreased by inhibition of glucosylceramide synthase (GCS) with the selective inhibitor PPPP. Inhibition of GCS, and thereby inhibited conversion of ceramides to glucosylceramides, abrogates the first step in ganglioside synthesis. Significantly decreased GD2-expression on PPPP-treated target cells (Fig. 3.3.6) resulted in significant abrogation of target cell lysis mediated by NK-92-scFv(ch14.18)-zeta (Fig. 3.3.7). This demonstrates a direct correlation between GD2-expression and NK-92-scFv(ch14.18)-zeta-mediated lysis. Additionally, the potential for GD2 alone to induce activation of NK-92-scFv(ch14.18)-zeta in absence of other activating stimuli was analyzed in a target cell-free experimental setting, based on immobilized GD2 (Fig. 3.3.4 and 3.3.5). Immobilized GD2 was sufficient to stimulate a significantly increased release of granzyme B and perforin, indicating activation of NK-92-scFv(ch14.18)-zeta. This effect was abrogated by blocking the CAR, confirming that activation is mediated by interaction of the CAR with immobilized GD2, which demonstrated that immobilized GD2 is sufficient to induce activation of NK-92-scFv(ch14.18)-zeta. This finding is particularly important, considering the general complexity and tight regulation of NK cell activation and that sometimes several activating receptors must be synergistically engaged to induce activation and cytotoxicity of NK cells. Importantly, these experiments demonstrate that recognition of GD2 by the CAR results in activating signaling, leading to the release of effector molecules. This suggests that GD2-specific lysis of NK-92-scFv(ch14.18)-zeta is actually due to activation of CAR signaling and not due to binding of the target cell via the CAR and activation via another ligand/receptor interaction. Additionally, these results indicate that targeting GD2 with the GD2-specific CAR expressed on NK-92-scFv(ch14.18)-zeta would allow the therapy to overcome escape mechanisms developed by cancer cells, such as shedding of activating ligands.

Based on the establishment of GD2-specificity and functionality of CAR-expressing NK-92-scFv(ch14.18)-zeta we could clearly show the primary role of the interaction of GD2 with the CAR for activation of NK-92scFv(ch14.18)-zeta and thereby NK-92-scFv(ch14.18)-zeta-mediated lysis. NK-92-scFv(ch14.18)-zeta were then used to address the problem of developing effective therapy against refractory relapse cell lines which remains a major challenge in high-risk NB treatment with conventional therapies. NB cell line pairs of four patients, each consisting of a cell line generated at time of diagnosis and a corresponding relapse cell line, were analyzed for their sensitivity towards NK-92-scFv(ch14.18)-zeta (Fig. 3.4.1). Importantly, all relapse cell lines were sensitive towards NK-92-scfv(ch14.18)-zeta-mediated lysis with three out of four relapse cell lines displaying even higher sensitivity than the corresponding cell line from time of diagnosis. This finding correlated with an

increased GD2-expression in the respective relapse cell lines (Fig. 3.4.2). Since it was shown that GCS, as the first enzyme in ganglioside synthesis has an impact on GD2 expression, GCS-expression in all cell line pairs was analyzed with Western blot (Fig. 3.4.3). This analysis revealed an increase in GCS-expression in three relapse cell lines compared to their corresponding cell line from diagnosis. The fourth relapse cell line displayed only slightly increased GCS-expression. While this last observation remains of interest, it would be necessary to analyze a greater number of cell line pairs to confirm this finding.

These encouraging *in vitro* results provided a baseline to test the therapeutic efficacy of NK-92-scFv(ch14.18)-zeta *in vivo* in a drug-resistant xenograft mouse model. This mouse model was based on induction of a subcutaneous human NB tumor followed by repeated peritumoral applications of NK cells, in combination with IL-2 to promote the survival of NK cells as well as to maintain high CAR-expression. Injection of NK cells was started on day three after tumor cell inoculation (Fig. 3.5.1). Since the idea of a cellular immunotherapy following conventional standard therapy is to treat minimal residual disease (MRD), which could otherwise potentially lead to relapse, NK cell injections were started at a time point with no palpable or visible signs of a tumor. Monitoring of tumor growth in all experimental groups revealed that palpable tumors appeared later in NK-92-scFv(ch14.18)-zeta-treated mice, and tumor growth rates were significantly delayed. Control groups displayed aggressive tumor growth, in contrast to NK-92-scFv(ch14.18)-zeta-treated mice, in which NK cell injections suppressed tumor growth in the majority of treated mice (Fig. 3.5.3 and 3.5.4). Mice in this NK-92-scFv(ch14.18)-zeta-treated group developed tumors after the end of NK cell treatment. Interestingly, tumors of mice receiving NK-92-scFv(ch14.18)-zeta already showed signs of necrosis at smaller tumor volumes than control groups and therefore had to be sacrificed before the maximal tumor volume was reached. These data provide a proof of concept that therapeutic application of NK-92-scFv(ch14.18)-zeta can mediate an *in vivo* anti-tumor effect towards an aggressively growing tumor and leads to significantly prolonged survival in mice receiving GD2-specific therapy (Fig. 3.5.5).

4.4. Toxicities and potential side effects of CAR-based approaches and strategies for circumvention

Despite its potential for cancer therapy, the use of genetically modified effector cells expressing a CAR can be accompanied by potential toxicities and side effects. On-target toxicities on healthy tissue are one major complication that should be considered. These toxicities most likely occur if healthy tissue also displays expression of the target antigen,

resulting in the destruction of healthy cells. Hence, target antigens for CAR-based immunotherapeutic approaches should be carefully chosen with high levels of expression on the tumor but restricted physiological expression. However, the desired on-target toxicity towards the tumor can also be accompanied by severe side effects. Strong activation of CAR-expressing effector cells, or activation of a high number of effector cells at the same time and subsequent destruction of tumor cells, may result in the release of high amounts of cytokines, such as TNF-α, IFN-γ, IL-2 or IL-6, leading to cytokine release syndrome (CRS). This can cause severe fever or hypotension and might result in life-threatening conditions, including multiple organ dysfunction [211-213]. Clinical applications of CAR-expressing T cells have reported the development of CRS as a side effect of adoptive transfer of CAR-expressing effector cells [197, 199].

Avoiding these toxicities and potential side effects is a major challenge for the therapeutic application of CAR-expressing effector cells. To address this problem of on-target toxicities towards healthy tissue, a new CAR approach is currently under investigation, based on the co-expression of an antigen-specific CAR and a chimeric co-stimulatory receptor (CCR) [214]. In this approach, an antigen-specific CAR that mediates only suboptimal activation is combined with a second receptor recognizing another antigen on the tumor and providing co-stimulation. Hence, effector cells only receive enough activation when both the CAR and the CCR interact with their respective antigen on the tumor. Engagement of only one of two receptors with their respective ligand provides only insufficient stimulation of effector cells. This potentially avoids toxicity towards healthy tissue when antigens are used that are not absolutely restricted to the tumor but are also expressed to some extent on healthy tissue [214]. Alternatively, the feasibility of a combination of an activating CAR recognizing a TAA and a second inhibitory CAR, specifically recognizing an antigen that is expressed on normal tissue, is under investigation to address the problem of on target, off organ toxicities [215]. In this approach, inhibitory CARs (iCARs) are based on the expression of inhibitory intracellular signaling domains of the immunoinhibitory receptors CTLA-4 and PD-1. Co-expression of activating CAR and iCAR was reported to result in inhibition of on-target cytotoxicity against other organs than the tumor when the iCAR encounters its respective antigen expressed on normal tissue [215].

Regarding the development of CRS, several aspects have to be considered. Since the extend of CRS has been shown to correlate with the disease burden [213, 216], the risk of CRS might be reduced by the restriction of CAR-based immunotherapy to a minimal residual disease setting. Further, the CAR-design might have an impact on potential side effects. Although improvement of CAR design based on the addition of co-stimulatory

domains might be beneficial in terms of stronger activation, prolonged persistence or increased proliferation of engineered effector cells, this might also require investigation into possible side effects related to strong activation or proliferation of TAA-specific effector cells. Even if the use of autologous effector cells might be beneficial in terms of low immunogenicity of CAR-expressing effector cells and therefore persistence of these cells, there is also the potential disadvantage of extensive proliferation of effector cells in response to TAA recognition on the tumor. This might result in increased numbers of effector cells reaching a threshold at which activation of these effector cells could potentially cause a CRS. To address this potential safety concern, suicide switches have been incorporated into the concept of adoptive transfer of CAR-expressing effector cells [217]. Safety switches based on the Herpes simplex virus thymidine kinase [218], inducible caspase 9 [219] and CD20 [220] have already been generated. These suicide switches enable elimination of CAR-expressing effector cells in the case of critical proliferation. The disadvantage of this approach would be that the effect is not some kind of preventive therapy and will be used when indications for too high effector cell numbers or pathologically elevated high cytokine levels are already detectable. Additionally, suicide gene-based induction of apoptosis would also completely abrogate the therapeutic effect of CAR-expressing cells.

Alternatively, the use of non-proliferating effector cells or effector cells which exhibit only transient CAR-expression, facilitated by transfection with mRNA encoding for the CAR, might be disadvantageous in terms of persistence of the anti-tumor effect but might be beneficial with regard to the safety of the therapeutic approach [137, 221, 222]. Irradiation can prevent proliferation of effector cells while maintaining cytotoxic activity. The advantage of non-proliferating effector cells is that the procedure of their therapeutic application would be comparable to the application of conventional therapeutic agents. Since the effector cells do not proliferate, the exact dosage can be calculated and dose escalation can be performed to determine a maximal tolerated dose that is not associated with severe side effects. Further, in case of unexpected adverse events, treatment can immediately be stopped by discontinuing the injection of cells or by halting additional effector cell injections. Therefore, the application of non-proliferating cells appears to be safer and more predictable. On the other hand, this approach may potentially require injection of higher cell numbers and repeated application of effector cells to maintain an anti-tumor response. Generation of sufficient numbers of CAR-expressing effector cells is therefore a crucial prerequisite. Due to recent improvements of protocols for NK cell isolation and *ex vivo* expansion [201-204], NK cells are an increasingly attractive effector cell population for CAR-based immunotherapeutic approaches. NK cells appear to be

advantageous with regard to their natural cytotoxicity especially towards tumor entities that lack MHC class I but still express activating NK cell ligands. NK cells also have an improved safety profile in an allogeneic setting, since they exhibit an increased cytotoxicity compared to the autologous setting but do not cause GvHD, a potential severe complication that can be caused by allogeneic T cells [177, 205, 206].

Unless endogenous T cell receptor genes are removed during genetic engineering, T cells still express their T cell receptor in addition to the TAA-specific CAR. When those T cells encounter their respective TAA recognized by the CAR, they get activated and proliferate. Expansion of this T cell population does not only mean expansion of TAA-specific CAR-expressing T cells, but also proliferation of T cells with a T cell receptor of unknown specificity, which might be another safety concern. Additionally, memory-like features of T cells compared to NK cells might be beneficial with regard to retaining a long-term anti-tumor response, but also bear the potential risk of long-term side effects if the TAA is not exclusively expressed on tumor tissue only [197, 198, 207].

Another aspect to be considered is the potential immunogenicity of the expressed CAR as a “foreign” protein. In the case of CAR-generation based on scFvs consisting of murine variable regions, there is the possibility that this might induce an immune response directed against the CAR, resulting in clearance of CAR-expressing effector cells from the system and thereby abrogation of the therapeutic activity. The development of anti-idiotype antibodies specifically binding the paratopes of the CAR has been reported in a number of cases [190, 223, 224], in contrast to others not showing the induction of anti-idiotype antibodies [189]. The use of scFvs that were derived from humanized antibodies for CAR generation might be beneficial in terms of lower potential to induce anti-idiotype antibodies. Interestingly, in the clinical application of murine GD2-specific antibodies, the induction of human anti-mouse antibodies (HAMA) correlated with improved outcome of patients [59]. This might be due to the induction of anti-anti-idiotype antibodies according to the anti-idiotype network theory [60], which recognize the nominal antigen GD2. In addition to these humoral immune responses, genetically engineered effector cells can also induce cell-mediated immune responses, which potentially limit therapeutic efficacy [223, 225].

The therapeutic approach presented in this study is based on the repeated application of a human NK cell line expressing a GD2-specific CAR (NK-92-scFv(ch14.18)-zeta). With regard to a clinical application, NK-92-scFv(ch14.18)-zeta can be irradiated to prevent proliferation. Parental NK-92 cells tested in clinical trials have been subjected to irradiation with 10 Gy prior to injection [124, 125], and the cytotoxicity of irradiated GD2-specific NK-92-scFv(ch14.18)-zeta has been shown to be comparable to non-irradiated NK-92-scFv(ch14.18)-zeta [150]. This is a prerequisite for the safety of the injection of a

human NK cell line into patients to avoid induction of a lymphoproliferative disease. Importantly, these cells can be expanded under GMP conditions to provide sufficient cell numbers for repeated injections during therapy, another prerequisite for a clinical application. So far, two clinical trials investigated the application of parental NK-92 cells in patients with advanced malignancies. The first phase I study analyzed the safety of NK-92 administration in 12 patients with renal cancer or melanoma based on a dose escalation including four different dose levels $1x10^8/m^2$, $3x10^8/m^2$, $1x10^9/m^2$ and $3x10^9/m^2$ [124]. Patients received three infusions of the respective NK-92 cell number within a 48 h infusion period on days 1, 3 and 5. NK-92 application was well tolerated, mainly accompanied only by grade 1 fever as well as grade 3 and grade 4 hypoglycemia observed in two patients only. The second phase I study was based on treatment of 15 patients with solid tumors/sarcomas or leukemia/lymphoma [125]. Patients were subjected to two NK-92 injections, 48 h apart from each other with a dose escalation including three different NK-92 dose levels ($1x10^9/m^2$, $3x10^9/m^2$, $1x10^{10}/m^2$). One patient received additional NK-92 infusions (5 infusions over a 6 month period). Importantly, NK-92 infusions were well tolerated, and analysis of persistence of NK-92 cells in two patients demonstrated that NK-92 was detectable in the circulation up to 48 hours after injection.

Addressing the problem of allogenicity of this cell line, and thereby potential immune reactions of the patient's immune system towards these cells, patients were selected for the clinical trial based on the absence of cross-match with NK-92. The first study revealed anti-HLA antibodies recognizing NK-92 in one out of two patients tested [124]. However, in the second study only one out of seven patients was tested positive for anti-HLA antibodies 1 and 4 weeks after NK-92 application. Although both studies aimed at investigating the safety of repeated NK-92 infusions, some responses have been seen in both studies [125]. The first study reported a minor response in a patient with metastatic melanoma and a mixed response in a patient with renal cell carcinoma [124]. In the second study, two patients with advanced lung cancer showed clinically significant mixed responses, and treatment of another patient with lung cancer resulted in stable disease for about two years [125]. These data demonstrated that repeated application of NK-92 is safe and well tolerated with evidence for clinical activity in patients with advanced cancer. Importantly, immune reactions towards NK-92 were rare, suggesting that a repeated application of NK-92 is feasible for treatment of advanced cancer. These data regarding the parental NK-92 cell line suggest that an application of the GD2-specific cell line NK-92-scFv(ch14.18)-zeta will have a similar safety and activity profile. Based on these considerations, the findings reported here provide an important baseline for the design of phase I/II clinical trials to

address tolerability of and response to NK-92-scFv(ch14.18)-zeta therapy in a highly challenging population of stage 4 NB patients.

4.5. Conclusion

In vitro as well as *in vivo* data generated within the present study provide definite evidence for specificity of NK-92-scFv(ch14.18)-zeta and efficacy of a GD2-targeted cellular approach against drug-resistant NB. Importantly, the interaction of the target antigen GD2 on tumor cells with the CAR was shown to be the primary mechanism of activation and subsequent tumor cell lysis mediated by NK-92-scFv(ch14.18)-zeta. The use of allogeneic GD2-specific NK-92-scFv(ch14.18)-zeta might further increase the therapeutic potential of an anti-GD2 immunotherapy. As a non-individualized cellular therapy, the use of NK-92-scFv(ch14.18)-zeta is independent from primary patient cells. Therefore, the present therapy could remain a therapeutic option for heavily pre-treated patients with poorly functioning primary immune cells or unfavorable conditions regarding FcγR affinity or HLA/KIR mismatch status. Importantly, this CAR-based cellular approach is likely to have an improved pain toxicity profile compared to GD2-specific antibodies, because CARs do not activate the complement system which is responsible for the pain side effects. One major benefit of the NK-92-scFv(ch14.18)-zeta cellular approach is that effector cells can be made available on demand, which is an important prerequisite to start this cell-based maintenance therapy subsequent to conventional therapy in a multicenter setting.

In conclusion, these considerations and the pre-clinical data generated in the present study provide an important rationale for the clinical investigation of tolerability and efficacy of NK-92-scFv(ch14.18)-zeta in phase I/II trials to further improve the outcome for highly challenging stage 4 NB patients.

5. Summary

Neuroblastoma (NB) is a solid extracranial childhood malignancy of neuroectodermal origin. About 50% of NB patients belong to the high-risk group characterized by poor prognosis. Standard multi-modal therapy is based on high dose chemotherapy and stem cell support followed by differentiation therapy with 13-cis-retinoic acid. Although this has improved the outcome of NB patients, treatment of high-risk NB remains challenging due to development of drug-resistant progressive disease or relapse that no longer responds to conventional therapy. This emphasizes the urgent need of alternative adjuvant therapy regimen.

Along this line, the potential of passive immunotherapy for NB treatment has recently been shown in a phase III clinical trial. Treatment of high-risk NB patients with a combination of a GD2-specific chimeric antibody (ch14.18), IL-2, GM-CSF and 13-cis-retinoic acid resulted in an increase in the two year event-free survival rate by 20%. Despite these promising results, about 30% of the patients still do not survive the disease, emphasizing the need for further optimization.

The Disialoganglioside GD2 is an established antigen for immunotherapy of NB, due to its high expression on almost all NB but restricted physiological expression in the brain and peripheral nerve fibers. Cellular therapy of NB with natural killer (NK) cells is especially appealing because MHC class I expression is absent or low in most NB, rendering this tumor sensitive to NK cell recognition according to the missing self hypothesis. Additionally, natural cytotoxicity of NK cells, mediated by interaction of activating receptors expressed on NK cells and their respective ligands on tumor cells, has been shown to play a role in lysis of NB cells. It is therefore tempting to assume that a combination of passive immunotherapy with GD2-specific antibodies and adoptive transfer of NK effector cells would result in an improved NB therapy. To achieve this goal an NK cell line expressing a GD2-specific chimeric antigen receptor was engineered: NK-92-scFv(ch14.18)-zeta. This chimeric antigen receptor (CAR) consists of a GD2-specific scFv-fragment, which was generated from ch14.18, and the CD3ζ-chain as intracellular signal-transducing domain.

Within this thesis, GD2-specificity of NK-92-scFv(ch14.18)-zeta as well as efficacy towards GD2-expressing NB cell lines, including relapse cell lines that exhibit partial or multidrug resistance were demonstrated. *In vitro* experiments utilizing an anti-idiotype antibody or a GD2-specific antibody blocking the CAR or the target antigen GD2, respectively, resulted in almost complete abrogation of NK-92-scFv(ch14.18)-zeta-mediated lysis of $GD2^+$ NB cell lines. This indicates that the interaction between the CAR and its target GD2 is the main mechanism of activation of NK-92-scFv(ch14.18)-zeta. This

finding was confirmed by additional experiments demonstrating that immobilized GD2 is sufficient to induce activation of NK-92-scFv(ch14.18)-zeta in the absence of other activating stimuli, and that experimental down-regulation of GD2 surface expression significantly decreased NK-92-scFv(ch14.18)-zeta-mediated lysis of $GD2^+$ NB cell lines.

Importantly, repeated application of NK-92-scFv(ch14.18)-zeta in combination with IL-2 significantly decreased tumor growth and prolonged survival of mice in an aggressively growing drug-resistant xenograft NB mouse model. This provides an important baseline for further optimization of this approach with respect to a clinical application. Supporting this strategy is the fact that the parental NK-92 cell line can be expanded under GMP conditions and has been demonstrated to be safe and well tolerated by patients in clinical trials. These findings and the results presented in the current study suggest that GD2-specific NK-92 has potential for a future clinical application as NB-specific effector cells that would be ready on demand in a standardized quality.

6. Zusammenfassung

Das Neuroblastom (NB) ist ein solider, extrakranieller Tumor neuroektodermalen Ursprungs, der sich im Kleinkindalter manifestiert. Etwa 50% aller Patienten gehören der Hochrisikogruppe an, welche mit einer schlechten Prognose assoziiert ist. Die multimodale Standardtherapie beinhaltet eine Hochdosischemotherapie mit anschließender Stammzelltransplantation und einer darauffolgenden Erhaltungstherapie mit 13-cis-Retinsäure. Die Behandlung der Hochrisikopatienten stellt hierbei eine besondere Herausforderung dar, da die Mehrheit zwar auf die initiale Standardtherapie anspricht, im weiteren Verlauf jedoch häufig eine fortschreitende Erkrankung oder ein Rezidiv entwickelt, welches oftmals Resistenzen gegenüber Chemotherapeutika aufweist.

Dies erfordert die Entwicklung neuer Alternativen zur Standardtherapie. Durch eine passive Immuntherapie, die sich gegen das NB-assoziierte Disialogangliosid GD2 richtet, konnte kürzlich die 2-Jahres-Ereignis-freie Überlebensrate von 46% auf 66% angehoben werden. Das Therapieprotokoll basierte dabei auf der kombinierten Gabe des GD2-spezifischen chimären Antikörpers ch14.18 mit IL-2, GM-CSF und 13-cis Retinsäure. Das Disialogangliosid GD2 ist bei dieser Erkrankung ein für die Immuntherapie bereits etabliertes NB-assoziiertes Antigen, das eine starke Expression auf nahezu 100% der NB-Tumoren aufweist, während sich die physiologische Expression auf das Gehirn und periphere Nervenbahnen beschränkt.

Verschiedene andere Ansätze, basierend auf der Induktion einer zellulären Immunität, werden verfolgt, jedoch zeichnen sich NB-Tumore häufig durch eine geringe oder fehlende Expression von MHC Klasse I Molekülen aus. Dies wiederum macht eine auf NK-Zellen basierende Therapie zu einem ebenfalls vielversprechenden Ansatz zur Behandlung dieser aggressiven Erkrankung. Dafür spricht die Tatsache, dass die Lyse von NB-Zellen durch verschiedene Mechanismen der natürlichen Zytotoxizität von NK-Zellen vermittelt werden kann.

Auf Grundlage dieser Erkenntnisse wurde eine NK-Zelllinie generiert, die einen GD2-spezifischen chimären Antigenrezeptor (*chimeric antigen receptor CAR*) exprimiert (NK-92-scFv(ch14.18)-zeta). Die Hauptbestandteile dieses CAR sind ein Einzelkettenantikörper, welcher die variablen Regionen des GD2-spezifischen Antikörpers ch14.18 enthält und die CD3ζ-Kette als signaltransduzierende Komponente. Die zelluläre Therapie mit NK-92-scFv(ch14.18)-zeta stellt damit eine Kombination der passiven GD2-gerichteten Immuntherapie und des adoptiven Transfers von NK-Zellen dar.

Im Rahmen der vorliegenden Arbeit konnte gezeigt werden, dass NK-92-scFv(ch14.18)-zeta in der Lage sind, auch Chemotherapie-resistente GD2-positive NB-

Zelllinien effektiv abzutöten. *In vitro* wurde nachgewiesen, dass eine Unterbrechung der Interaktion zwischen dem CAR und GD2, die sowohl durch die Blockierung des CAR als auch durch Blockierung von GD2 hervorgerufen werden kann, zur fast vollständigen Aufhebung der NK-92-scFv(ch14.18)-zeta-vermittelten Lyse GD2-exprimierender NB-Zelllinien führt. Dies zeigt, dass die Erkennung und Bindung von GD2 durch den CAR der Hauptmechanismus der NK-92-scFv(ch14.18)-zeta-vermittelten Lyse ist. Weiterhin konnte demonstriert werden, dass immobilisiertes GD2 als Stimulus für die Aktivierung von NK-92-scFv(ch14.18)-zeta ausreichend ist und dass eine experimentell induzierte Verringerung der GD2-Expression zu einer verminderten Lyse der Zielzellen führt. Die Analyse von NB-Zelllinienpaaren hat außerdem ergeben, dass auch Zelllinien, die aus Rezidivtumoren generiert wurden, sensitiv gegenüber einer NK-92-scFv(ch14.18)-zeta-vermittelten Lyse sind und zum Teil sogar eine höhere Sensitivität aufweisen als entsprechende Zelllinien, welche zum Diagnosezeitpunkt aus dem gleichen Patienten entwickelt wurden.

Abschließend wurde die Effektivität von NK-92-scFv(ch14.18)-zeta darüber hinaus *in vivo* in einem Chemotherapie-resistenten $GD2^+$ *Xenograft*-Mausmodell gezeigt. Die wiederholte therapeutische Applikation von NK-92-scFv(ch14.18)-zeta in Kombination mit IL-2 resultierte dabei in einem signifikant verlangsamten Tumorwachstum und einem verbesserten Überleben im Vergleich zu den Kontrollgruppen.

Die Ergebnisse dieser Arbeit belegen, dass GD2-spezifische NK-92 das Potential für eine zukünftige klinische Anwendung besitzen. Bezüglich der Umsetzbarkeit einer solchen zellulären Therapie konnten klinische Studien zum adoptiven Transfer der parentalen Zelllinie NK-92 bereits zeigen, dass NK-92 in ausreichender Menge unter GMP-Bedingungen expandiert werden können und dass die Infusion der Zellen sicher und gut verträglich ist. Demnach stellt der Einsatz einer solchen GD2-spezifischen NK-Zelllinie, die zu jeder Zeit in einer standardisierten Qualität verfügbar wäre, eine vielversprechende Alternative zur Behandlung von Hochrisikopatienten dar, deren Erkrankung nicht mehr auf die Standardtherapie anspricht.

7. References

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8. Appendix

8.1. List of figures

8.2. List of tables

8.3. Publications

8.3.1. Papers

- **Seidel,D.**, Shibina,A., Siebert,N., Wels,W.S., Reynolds,C.P., Huebener,N., Lode,H.N. *Disialoganglioside-specific human natural killer cells are effective against drug-resistant neuroblastoma.* Cancer Immunol Immunother. 2015. *in press*.
- Stermann,A., Huebener,N., **Seidel,D.**, Fest,S., Eschenburg,G., Lode,H.N. *Targeting of MYCN by means of DNA vaccination is effective against neuroblastoma.* Cancer Immunol Immunother. 2015. *in revision*.
- Siebert,N., **Seidel,D.**, Eger,C., Jüttner,M., Lode,H.N. *Functional bioassays for immune monitoring of high-risk neuroblastoma patients treated with ch14.18/CHO anti-GD2 antibody*. PLoS. One. 9 .2014. e107692.
- Siebert,N., Eger,C., **Seidel,D.**, Jüttner,M., Lode,H.N. *Validated detection of human anti-chimeric immune responses in serum of neuroblastoma patients treated with ch14.18/CHO*. J Immunol Methods. 2014. 407:108-115.
- Siebert,N., **Seidel,D.,** Eger,C., Brackrock,D., Reker,D., Schmidt,M., and Lode,H.N., *Validated detection of anti-GD2 antibody ch14.18/CHO in serum of neuroblastoma patients using anti-idiotype antibody ganglidiomab*. J.Immunol Methods. 2013. **398-399**: 51-59.
- Lode,H.N., Schmidt,M., **Seidel,D.,** Huebener,N., Brackrock,D., Bleeke,M., Reker,D., Brandt,S., Mueller,H.P., Helm,C., and Siebert,N., *Vaccination with anti-idiotype antibody ganglidiomab mediates a GD(2)-specific anti-neuroblastoma immune response*. Cancer Immunol.Immunother. 2013. **62**: 999-1010.
- Shibina,A., **Seidel,D.**, Somanchi,S.S., Lee,D.A., Stermann,A., Maurer,B.J., Lode,H.N., Reynolds,C.P., and Huebener,N., *Fenretinide sensitizes multidrug-resistant human neuroblastoma cells to antibody-independent and ch14.18-mediated NK cell cytotoxicity.* J.Mol.Med.(Berl) 2013. **91**: 459-472.
- Esser,R., Muller,T., Stefes,D., Kloess,S., **Seidel,D**., Gillies,S.D., Aperlo-Iffland,C., Huston,J.S., Uherek,C., Schonfeld,K., Tonn,T., Huebener,N., Lode,H.N., Koehl,U., and Wels,W.S., *NK cells engineered to express a GD2 -specific antigen receptor display built-in ADCC-like activity against tumour cells of neuroectodermal origin.* J.Cell Mol.Med. 2012. **16**: 569-581.

8.3.2. Oral presentations

2014

- 8th Rostocker Symposium for Tumor Immunology in Childhood, Rostock, Germany

2012

- 4th International Tübingen-Symposium on Pediatric Solid Tumors, From Bench to Bedside in Neuroblastoma, Tübingen, Germany
- 6th Rostocker Symposium for Tumor Immunology in Childhood, Rostock, Germany

2011

- Central European Society for Anticancer Drug Research (CESAR) Annual Meeting 2011, Greifswald, Germany
- XXIV. Annual Meeting, Kind-Philipp-Stiftung für Leukämieforschung, Wilsede, Germany
- 5th Rostocker Symposium for Tumor Immunology in Childhood, Rostock, Germany

2009

- XXII. Annual Meeting, Kind-Philipp-Stiftung für Leukämieforschung, Wilsede, Germany
- 3th Rostocker Symposium for Tumor Immunology in Childhood, Rostock, Germany

8.3.3. Poster presentations

2014

- Advances in Neuroblastoma Research (ANR) Conference, Cologne, Germany
- American Association for Cancer Research (AACR) Annual Meeting 2014, San Diego, CA, USA

2012

- 3rd International Conference on Immunotherapy in Pediatric Oncology, Frankfurt, Germany
- Advances in Neuroblastoma Research (ANR) Conference, Toronto, Canada

2011

- American Association for Cancer Research (AACR) Annual Meeting 2011, Orlando, Florida, USA

2010

- Innovations in Cancer Research Prevention and Research Conference, Austin, Texas, USA
- American Association for Cancer Research (AACR) Annual Meeting 2010, Washington DC, USA
- Student Research Week 2010, TTUHSC, Lubbock, Texas, USA

2009

- 4th European Pediatric Congress EUROPEDIATRICS 2009, Moscow, Russia
- 4th ENII-Mugen Imunology Summer School, Capo Caccia, Sardinia, Italy
- AEK 15th International Cancer Congress, Berlin, Germany

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Ort, Datum Diana Seidel

8.4. Scholarships and awards

05/2014

- Travel scholarship, GlaxoSmithKline Foundation

04/2014

- AACR Scholar in Training Award

06/2012

- Travel scholarship, Kind-Philipp-Stiftung für Leukämieforschung

05/2011-05/2012

- Training grant, Kind-Philipp-Stiftung für Leukämieforschung

04/2011

- Travel scholarship, Kind-Philipp-Stiftung für Leukämieforschung

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Ort, Datum Diana Seidel

8.5. Versicherung an Eides Statt

Hiermit erkläre ich, die vorliegende Arbeit selbstständig und ohne unerlaubte Hilfe verfasst zu haben und alle Hilfsmittel und Inhalte aus anderen Quellen als solche gekennzeichnet zu haben. Des Weiteren versichere ich, dass die vorliegende Arbeit nie Gegenstand eines früheren Promotionsverfahrens war.

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Ort, Datum Diana Seidel